UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Territorio e Sistemi-Agroforestali

SCUOLA DI DOTTORATO DI RICERCA IN: Territorio, Ambiente, Risorse e Salute
INDIRIZZO: Medicina ambientale: Nutrizione e Inquinamento
CICLO: XXV

TITOLO TESI

Enzymatic characterization of two plant coagulants: *Cynara cardunculus* L. and *Ficus carica* L.

Caratterizzazione enzimatica di due coagulanti vegetali: Cynara cardunculus L. e Ficus carica L.

Direttore della Scuola: Ch.mo Prof. (Mario Aristide Lenzi)
Coordinatore d'indirizzo: Ch.mo Prof. (Giovanni Battista Bartolucci)
Supervisore: Ch.ma Dott.ssa (Giovanna Lomolino)

Dottoranda: Giovanna Di Pierro

Acknowledgements

Firstly I would like to thank my supervisor, Doct. Giovanna Lomolino, for giving me the opportunity to carry out this PhD. I also wish to thank Prof. Anna Lante for her help in any situations. Similarly, I would like to acknowledge Mara Vegro and Stefania Zannoni for their friendly and technical assistance at multiple occasions, and the students who participated in my research: Alvise da Schio and Alice Quaresima. I would like to express my gratitude to Prof. Gianfranco Gabai for getting me in contact with my Irish supervisor Prof. Richard J. FitzGerald, who allowed me to join the research on bioactive peptides. My special words of appreciation are for his helpful and valuable discussions about the experiments, and his critical review of part of this manuscript. To the entire staff of the "Department of Life Sciences" of the University of Limerick (Ireland), where I spent 10 wonderful months. Thank to Martina O'Keefe and Orla Power for their scientific support and my Irish collegues and dear friends: Roseanne Norris, Tara Flaherty, Elizabeth Finnegan, Bryan Hunt, for sharing with me the best moments of this adventure. The time spent in Limerick and around would definitely have been less pleasant and joyful without you. To the other people of the lab, Adriana, Tanya, Charlie Pigott and his brilliant music. A special thank to my Irish mum, Annette Norris, for taking care of me. To all the other PhD students and colleagues that shared with me these three years: Lucia Fiorin, Lucia Michelini, Chiara Rasotto and Federica Tinello. This list would not be complete without all the most important people around me: my big family and friends, in particular Laura, Silvia & Silvia and Valentina. Last but not least, a special thank to Andres for his love and his priceless support in every moment.

Thesis summary

The present thesis aims to enhance current knowledge of plant coagulants, whose interest is increasing because of the limited supply of calf rennet and the recent food trends. At this purpose, *Cynara cardunculus* L. and *Ficus carica* L., two crops widely distributed in Mediterrean countries, were selected and characterized in their enzymatic activity. At first, the presence of lipases in these crude plant extracts was investigated by employing agar plate and chromogenic assays as well as the zymogram analysis. Secondly, proteolytic activity was studied with the azocasein assay. Then, the hydrolysis of bovine α_s and β-caseins was examined by eletrophoretic gels and densitogram analysis in order to assess the contribution of each plant enzyme to the overall proteolytic specificity. Finally, both plant extracts were evaluated for their potential release of antioxidant peptides/hydrolysates from bovine milk casein. The results suggested the use of *Cynara cardunculus* L. and *Ficus carica* L. crude extracts in cheese making as sources of lipases, proteinases and bioactive peptides.

Riassunto

La presente tesi di dottorato intende approfondire le attuali conoscenze scientifiche in relazione ai coagulanti vegetali, il cui interesse è in continuo aumento a causa della limitata disponibilità di caglio animale e i correnti trend alimentari. A tale scopo, *Cynara cardunculus* L. e *Ficus carica* L., due specie vegetali largamente diffuse nei paesi mediterranei, sono state scelte e caratterizzate nelle loro attività enzimatiche. Dapprima, si è verificata la presenza di lipasi in questi estratti vegetali attraverso test in piastra su agar, substrati cromogenici ed analisi zimografica. Successivamente, si è caratterizzata l'attività proteolitica con il saggio dell'azocaseina. Al fine di studiare il contributo di ciascuna di queste proteasi vegetali sulla specificità proteolitica, l'idrolisi dell'α_s- e β-caseina è stata monitorata attraverso gel elettroforetici e l'analisi densitometrica. Infine, questi stessi estratti vegetali sono stati valutati per il loro potenziale rilascio di peptidi/idrolizzati antiossidanti da caseine bovine. Pertanto, gli estratti di *Cynara cardunculus* L. e *Ficus carica* L. possono trovare impiego nel settore lattiero-caseario come fonti di lipasi, proteasi e biopetidi attivi.

List of abbreviations

APs: aspartic proteinases;

AU: arbitrary units;

AUC: area under curve;

α_s-CN: α_s-casein;

BAPs: bioactive peptides;

BSA: bovine serum albumin;

βNa: β-naphtyl acetate;

βNb: β-naphtyl butyrate;

β-CN: β-casein;

βNp: β-naphtyl proprionate;

CC: Cynara cardunculus;

CPs: cysteine proetases;

CS: clear supernatant;

DH: degree of hydrolysis;

D-PAGE: deriphat polyacrylamide gel electrophoresis;

FDA: fluorescein diacetate;

FDB: fluorescein dibutyrate;

FDC: fluorescein dicaproate;

FDL: fluorescein dilaurate;

FFA: free fatty acid;

FL: fluorescein;

FU: fluorescence units;

GM: gummy material;

GP-HPLC: gel permeation high pressure liquid chromatography;

Na-CN: sodium caseinate;

N-PAGE: native polyacrylamide gel electrophoresis;

ORAC: oxygen radical absorbance capacity;

pNa: p-nitrophenyl acetate;

pNb: p-nitrophenyl butyrate;

pNp: p-nitrophenyl propionate;

RFI: relative fluorescence intensity.

TABLE OF CONTENTS

CHAPTER 1

Plant coagulants: an overview

ABSTRACT

The use of plant coagulants in cheese production is known since ancient times. Many plant extracts have milk-clotting activity, such as cardoon (*Cynara cardunculus* L.), fig (*Ficus carica* L.), papaya (*Carica papaya*) and pineapple (*Ananas comosus*). Despite the limitations deriving from their use in cheese-making (bitter taste and texture defects), recently there is a growing interest in vegetable coagulants as an alternative to traditional calf rennet. Moreover, current food trends, due to dietary (vegetarian) and religion restrictions (Islamism and Judaism), are leading to the use of these plant extracts. Therefore, further studies need to be carried out to enhance current knowledge of plant coagulants.

Keywords: animal rennet, microbial coagulants, plant coagulants, *Ficus carica* L., *Cynara cardunculus* L.

INTRODUCTION

Rennet and coagulants are preparations of proteolytic enzymes, some of which have been used in cheesemaking for thousands of years, and they seem to be the oldest known application of enzymes (Harboe *et al.*, 2010). The first name for the milk-clotting enzyme was chymosin, derived from the Greek word for gastric liquid *chyme*, given by Deschamps (1840) to the main enzyme from the fourth stomach of the calf. In 1890, the name rennin, derived from the word rennet, was suggested for the same enzyme, and for many years it was adopted in English-speaking countries (Foltmann, 1966), as well as in international enzyme nomenclature. Due to confusion with the related proteolytic enzyme renin, the main milk-clotting enzyme was again named chymosin (International Union of Biochemistry and Molecular Biology – IUBMB, 1992). Cheese is produced by milk-clotting enzymes of different origin. The active milk-clotting enzymes in all rennet and coagulants, which have

been found successful for cheesemaking, are aspartic proteinases having the IUBMB number EC 3.4.23, which specifically cleave the Phe_{105}-Met_{106} bond of bovine κ-casein. The original rennet preparation is, by definition, an extract of ruminant abomasum (Andrén, 1998), which is commonly called animal rennet. This definition is now generally accepted, and it is agreed that the name *rennet* should be reserved for enzyme preparations from ruminant stomachs, whereas other milk-clotting enzymes should be named *coagulants*. The common group names are microbial and vegetal coagulants, respectively. It is also generally accepted that chymosin produced by a genetically modified organism (GMO) is called *fermentation-produced chymosin* (FPC).

The present chapter gives an overview of the relevant enzymes in cheesemaking, with special emphasis on plant coagulants. The main important plant coagulants are described in their enzymatic composition along with their research background. A historical overview is also given. Furthermore, the impact of these plant coagulants on cheese yield, texture and sensory properties as well as their ethical and religious influence are discussed in detail. Due to the increasing demand for alternative sources of animal rennet and current food trends, the presents thesis intends to characterize the two plant coagulants, *Ficus carica* L. and *Cynara cardunculus* L, respectively. Finally, the scientific objectives of the thesis are defined in order to enhance the scientific information on these plant coagulants and promote their use in cheesemaking.

1. TYPES OF RENNET AND COAGULANTS

Rennet and coagulants are most efficiently categorised according to their sources, which are animal, microbial and genetically engineered chymosin as well as plant-derived clotting enzymes. The types of rennet and coagulants as well as their characteristics have been reviewed by several authors (Harboe, 1992a; Guinee and Wilkinson, 1992; Garg and Johri, 1994; Wigley, 1996, Jacob *et al*., 2011). The following paragraphs describe the predominant types of coagulant used for cheesemaking today, with special emphasis placed on plant coagulants.

1.1 ANIMAL RENNET

Most of cheeses are traditionally manufactured by rennet extracted from the abomasum, the fourth stomach of young ruminants. Among the products of animal origin, calf rennet is regarded as the ideal enzyme product for cheesemaking; this extract consists of two proteolytic enzymes: chymosin (EC 3.4.23.4, 88-94% milk clotting activity MCA) and pepsin

(EC 3.4.23.1, 6-12% MCA). The relative proportion of these enzymes varies with individuality and age of the animals, the type of feed, the method of rennet separation and the conditions and pH values at which the milk clotting activity is measured (Guinee and Wilkinson, 1992; Andrèn, 1982). As previously defined, the main role of chymosin in cheesemaking is to coagulate milk by specifically hydrolysing the Phe_{105}-Met_{106} bond of the micelle-stabilising protein, κ-casein, which is more susceptible to chymosin than any other bond in milk proteins and leads to the coagulation of the milk (Fox *et al*., 2000); whereas pepsin is much less specific and hydrolyses bonds with Phe, Tyr, Leu or Val residues (Agudelo *et al*., 2004; Papoff *et al*., 2004). The traditional product, calf rennet, is, still now, the reference product used as control when other coagulants are tested. Adult bovine rennet is the most widely used alternative to calf one, which is not surprising as it contains the same active enzymes. The high pepsin content in adult bovine rennet gives the product a high sensitivity to pH, and a higher aspecific proteolytic activity. Several other products exist, of these lamb/ovine and kid-caprine/caprine rennet are very similar to calf/adult bovine rennet, but they are best suited for clotting milk of their own species (Foltmann, 1992). Buffalo, chicken, rabbit and swine have also been found to be sources of rennet (Dewane, 1960).

1.2 MICROBIAL COAGULANTS

A large number of microorganisms are known to produce milk clotting enzymes. Most of the coagulants from microbial sources seem to be extracellular (Garg and Jhori, 1994) and since their action is similar to chymosin, they are suitable for cheese production. These enzymes show, however, high proteolytic activity during cheesemaking, which may lead to a loss of protein degradation products into the whey and thus negatively affect cheese yield (Jacob *et al*., 2011). At present the main important microbial coagulants are of fungal and bacterial origin.

1.2.1 FUNGAL COAGULANTS

Microbial coagulants of fungal origin, which have been used in commercial cheesemaking since the 1960s, and are considered of great importance. More than 100 fungal sources were reported by Garg and Johri (1994), which points out the high scientific interest in alternative coagulants for cheese production. Fungi producing milk clotting proteinasess are ubiquitary and may easily be isolated from various environments (Tubesha and Al-Delaimy, 2003). Three species, *Mucor mihei* (*Rhizomucor miehei*), *Mucor pusillus (Rhizomucor pusillus*) and *Endothia parasitica* (*Criphonectria parasitica*), have been tested

on large scale production. In general, the rennet produced by fungi is an acid-aspartate proteinase with a single polypeptide chain. The aspartic proteinase produced by *R. miehei* has a high similarity to chymosin (Chitpinityol and Crabbe, 1998) and is the most commonly used microbial coagulant for cheese production and commercially available at different levels of thermostability and purity. The *R. pusillus* coagulant is similar to the *R. miehei* product; it was used in the past, but has no advantages rather than *R. miehei* coagulant, and is no longer produced commercially (Harboe *et al.*, 2010). *Cryphonectria parasitica* proteinase is less characterised, but it is well known that its general proteolytic activity is higher (Tam and Whitaker, 1972; Vanderporten and Weckx, 1972) toward β-casein (Ustunol and Zeckzer, 1996; Awad *et al*., 1998, 1999; Trujillo *et al*., 2000; Broome *et al*., 2006).

1.2.2 BACTERIAL COAGULANTS

Besides enzymes from other fungal species, enzymes of bacterial origin have gained increasing importance (Jacob *et al*., 2011). However, most of the bacterial proteinases have been found to be unsuitable in cheese production, because of the invariably strong and non specific proteolytic action of the milk-clotting enzymes, resulting in loss of fat and nitrogen in the whey, reduced yield and poor quality of the aged cheese (Garg and Jhori, 1994). In addition, many of these bacteria are known to be pathogenic. Many studies were carried out on bacterial proteinases (Srinivasan *et al*., 1962; Orosin *et al*., 1970; Sardinas, 1972; Puhan and Irvine, 1973; Reps *et al*., 1974). *Bacillus prodigiosus* was one of the first identified bacteria to produce milk clotting enzymes (Gorini, 1892, 1893, 1930; Wahlini, 1928). *Bacillus polymyxa*, *B. subtilis*, *B. cereus* and *B. mesentericus* were mentioned as other sources of proteinases, but all of them produced defects on texture and flavor of final cheese. At present there is no commercially available bacterial coagulants which substitute animal rennet. They are applied for particular cheeses or as partial substitutes in mixture with other enzymes, such as pepsin, animal rennet, or with other microbial rennets (Garg and Jhori, 1994).

1.2.3 GENETICALLY ENGINEERED CHYMOSIN

In 1990 the recombinant version of calf chymosin, usually denoted as fermentation produced chymosin (FPC), was the first proteinase, obtained by recombinant DNA, applied by FDA in USA (Flamm, 1991). At the beginning, was used in the United States, but later other countries showed increasing acceptance. FPC is chymosin produced by fermentation of a genetically modified organism (GMO). The products contain chymosin identical to the

animal source, which has with the same amino acid sequence as chymosin from the corresponding animal stomach, but it is just produced by more efficient means. The main FPC, which contains bovine chymosin B, is today considered to be the ideal milk-clotting enzyme against which all other milk-clotting enzymes are compared. The production and application of bovine-type FPC has been reviewed by several authors (Harboe, 1992b, 1993; Repelius, 1993).

1.3 PLANT COAGULANTS

Plant coagulants can be an interesting alternative to the animal rennet for several reasons. Firstly, the worldwide increase in cheese production, a greater tendency to slaughter "mature" calves along with high costs and the limited supply of calf rennet, have led to an increase in the demand for alternatives sources of milk coagulants (Green, 1972; Cavalcanti *et al*., 2004). Secondly, the incidence of bovine spongiform encephalopathy has further on reduced the demand for bovine rennet (Roseiro, *et al*., 2003). Finally, plant coagulants can contribute to improve the nutritional intake of people whose the use of animal rennets is restricted (Gupta and Eskin, 1977).

Table 1 Sources of plant coagulants.

Plant source	Common name	References
Cynara cardunculus	Cardoon	Veríssimo *et al* ., 1995, 1996
Cynara scolymus	Artichocke	Sidrach *et al* ., 2004
Ficus carica	Fig tree	Devaraj *et al* ., 2008
Carica papaya	Papaya	Veringa, 1961
Ananas comosus	Pineapple	Bruno *et al* ., 2002, 2010
Albizia julibrissin	Silk tree	Otani *et al* ., 1991
Calotropis procera	Sodom apple	Ibiama and Griffiths, 1987
Lactuca sativa	Lettuce	Lo Piero *et al* ., 2002

Therefore, these reasons have encouraged a search for new rennet sources, like plant coagulants. In this regard, many enzymes from plants have been found to coagulate milk (Table 1) and several authors have been investigated their use in cheese production (Padhmanabhan *et al*., 1993; Cattaneo *et al*., 1994; Teixeira *et al*., 2000; Fadyloglu, 2001; Patil *et al*. 2003; Llorente *et al*., 2004; Moharib, 2004; Libouga *et al*., 2006; Low *et al*., 2006; Senthilkumar *et al*., 2006; Chazarra *et al*., 2007; Egito *et al*., 2007; Raposo and Domingos, 2008; Vairo Cavalli *et al*., 2008; Duarte *et al*., 2009). These enzymes seemingly ubiquitous in

plant structure. They are present in buds, flowers, fruits, latex, leaves, roots, sap and seeds (Garg and Jhori, 1994).

1.3.1 HISTORICAL OVERVIEW

Since ancient times, milk clotting enzymes have been used for the manufacture of cheese. Historically, most enzyme preparations used for cheese have been extracted from the stomachs of ruminants, but coagulants from microbes and plants were also used at very early dates. Vegetable rennets are mentioned in the first work of European literature; Homer (c. eighth century BC) implies the use of fig rennet in the *Iliad* ('. . . as when fig juice is added to white milk and rapidly coagulates, and the milk quickly curdles as it is stirred, so speedy was his healing of raging Ares.' *Iliad* **5**) (Fox and McSweeney, 2004), while in the *Odysse*y, he describes the Cyclops, Polyphemus, making curds using 'fig juice' (*Ficus carica* L.) (Salvadori del Prato, 1998). Cheese manufacture was well established in the Roman Empire and many writers, e.g., Cato the Elder, Varro, Columella and Pliny the Elder, described cheese manufacture and quality and the culinary uses of cheese. Varro (116-27 BC) mentioned fig latex as an alternative rennet in *De re pecuaria*. However, the most complete ancient description of cheesemaking is given by Lucius Junius Moderatus Columella, a Roman soldier and author from Gades (moder Cadiz) (Fox and McSweeney, 2004). He discusses different types of rennets in his treatise *De Re Rustica* (50 AD), by describing in detail their use, doses and technology production of cheese (Salvadori del Prato, 1998). Furthermore, he recommends coagulation using rennet from lamb or kid but states that milk can also be coagulated using flowers of certain thistles (perhaps *Cynara cardunculus*), seeds of the safflower (*Carthamus tinctorius*), or sap from the fig tree ('. . . though it can also be coagulated with the flower of the wild thistle or the seeds of the safflower, and equally well with the liquid which flows from a fig-tree.') (Fox and McSweeney, 2004; Robinson and Wilbey, 2008). Interestingly, Columella recommended that the smallest amount of rennet possible be used to ensure high quality cheese. This may be related to the excessive proteolytic activity of plant proteinases used as rennets which often produce bitter cheese (Fox and McSweeney, 2004). According to Roman writers, plant coagulant such as the latex of fig (*Ficus carica* L.), flowers and flowers heads of thistle (*Cynara cardunculus* L.) and in particular way inflorescences of gallium (*Gallium verum*, a plant of Family *Rubiaceae*) were more suitable for the production of soft cheese; while rennet (obtained from the stomach of ruminants) was more suggested for the production of *formaticum* or hard cheese. In the Middle Ages plant coagulants were used largely, such as thistle flowers, fig latex, pumpkin,

drops of balsam and calin thistle (*Carlina aucalis*). In XVII century, Ignazio Malenotti recommended in the *Handbook of shepherd* to leave the use of animal rennet in favour of *presura*, the curd obtained by the flower of wild artichoke (Salvadori del Prato, 1998).
Nowadays, cheeses made with vegetable coagulant can be found mainly in Mediterranean, west African and southern European countries. Spain and Portugal have the largest variety and production of cheeses using *Cynara* sp. as the vegetable coagulant (Roseiro *et al*., 2003).

1.3.2 CYNARA CARDUNCULUS L.

Cynara cardunculus L. is a perennial herb native to the Mediterranean region, grown since ancient times as a vegetable using intensive management techniques. It belongs to the family of the Asteraceae-Cardueae, containing eight species and four subspecies, including the well-known globe artichoke and the cardoon. The adult plant of *Cynara cardunculus* in its natural growth cycle exhibits vigorous growth; a single plant can reach a height of 3 m and spread over an area of 1.5 m in diameter (Roseiro *et al*., 2003; Fernández *et al*., 2006). The clotting properties of cardoon extracts were first studied by Christen and Virasoro (1935), who established that the enzymatic activity was only present in the flower and it was maintained at temperatures up to 70°C. Subsequent investigations confirmed these results (Vieira de Sá and Barbosa, 1972; Tsouli, 1974), the enzyme activity being traced to the stylets and stigmae of the inflorescence (Faro, 1991; Ramalho-Santos *et al*., 1997). The aqueous extract of flowers of *C. cardunculus* was further shown to possess three active proteinases (once termed cynarases or cyprosins, and currently termed cardosins) which have been isolated, purified and partly characterized in terms of activity (Heimgartner *et al*., 1990; Cordeiro *et al*., 1992) and specificity (Macedo *et al.*, 1993; Macedo, 1993; Pires *et al*., 1994) toward pure bovine caseins. More recently, two additional aspartic proteinases were isolated from the fresh stigmae of a standard variety of *C. cardunculus* L. Based on the structural and kinetic properties of these enzymes, it was concluded that they result from different genes and are different from the previously reported proteinases of the same plant; hence, they were named cardosin A and cardosin B (Faro *et al.*, 1995; Verissimo *et al*., 1996). Each cardosin consists of two subunits with apparent molecular weights of 31 and 15 kDa for cardosin A and 34 and 14 kDa for cardosin B (Verissimo *et al*., 1995, 1996). Esteves (1995) showed that cardosin A appears to be similar to chymosin; in fact it cleaves the bond Phe_{105}-Met_{106} in bovine κ-casein, whereas cardosin B closely resembles pepsin. However, other authors reported the purification of three milk clotting enzymes from dried flowers of *Cynara cardunculus* L. (Heimgartner *et al*., 1990; Cordeiro *et al*., 1992). These enzymes were named

cynarases and subsequently cyprosins and were assumed too have been derived from a common precursor by different processing (Cordeiro *et al*., 1994). Cyprosins have been showed to differ significantly from any of the cardosins (Veríssimo *et al*., 1996). Overall, the aspartic proteinases of wild cardoon are the most studied enzymes in vegetable kingdom, because of their excellent and clotting properties; moreover, they have a specific proteolytic action. This could be one of the reasons for which the extracts of cardoon have been used for centuries in traditional artisanal production of ewe milk cheeses, such as Serra da Estrela, Manchego, La Serena or Serpa in Portugal and Spain. However, the availability of plant material is seasonal, presents a considerable variability in enzyme concentration, largely depending on the harvesting region and on the climatic conditions of the year (Pais, 2002).

1.3.3 CYNARA SCOLYMUS L.

Cynara scolymus L., also known as the globe artichoke, is another variety belonging to the family of Asteraceae. It is often confused with *Cynara cardunculus* L. and it is widely distributed in the Mediterranean regions. Globe artichokes are perennial, frost sensitive, thistle-like plants with edible flower buds, which sprout from the terminal portion of the main stem and on lateral stems. Each unopened flower bud resembles a deep green pine cone, 7–10 cm in diameter, round, but slightly elongated. Several pointed, leathery green bracts fold around a purple-blue flower. The base of each bract is the fleshy edible portion, along with the fleshy centre of the artichoke on which the flower and bracts are borne (Sidrach *et al*., 2005). The extracts of the flowers of this specie have been claimed to be effective as rennet (Silva and Malcata, 2000; Veríssimo *et al*., 1998), but because the flowers of this plant are usually consumed as a vegetable, the properties and possible applications of these cynarases are less known Recently, Chazarra *et al*. (2007) have purified three proteinases of *Cynara scolymus* L. (cynarase A, B and C) with milk clotting activity. All three cynarases are glycoproteins and are composed of one large and one small subunit (Sidrach et al., 2005).

1.3.4 FICUS CARICA L.

Fig (*Ficus carica* L.) is a fruit crop species that is particularly well suited for the different environmental conditions of the Mediterranean basin countries, and more 600 cultivars are locally grown and called varieties. *Ficus carica* L., the common fig, is a species of great commercial importance, comprising numerous varieties with significant genetic diversity (Salhi et-Hannachi *et al*., 2006). Other notable species of *Ficus* are *Ficus religiosa* L. (the Bo tree which sheltered the Buddha as he divined the “Truths”), *Ficus elastica* Roxb.

ex Hornem. (the rubber tree), *Ficus benghalensis* L. (the banyan tree) and *Ficus racemosa* L. (syn. *glomerata*, the giant cluster tree). All *Ficus* spp. possess latex-like material within their vasculatures, affording protection and self-healing from physical assaults (Lansky *et al.*, 2008). *Ficus carica* L. is known to coagulate milk and its latex constitutes an important source of many proteolytic enzymes known under the general term of ficin (EC 3.4.22.3). The name ficin was coined by Robbins in 1930 and it regarded a protein powder he prepared from the latices of fig trees of the genus *Ficus*. Ficin, which belongs to the cysteine proteinases, contains two groups of proteolytic enzymes; the first group includes high milk clotting activity but low proteolysis, and the second encloses high proteolytic effect (Whitaker, 1972). In several research studies (Sgarbieri *et al*., 1964; Devaraj *et al*., 2008; Azarkan *et al*., 2011) proteinases from *Ficus carica* have been purified and characterized, confirming the existence of multiple ficin forms in its latex. However, only a limited number of investigations have reported on the use of this plant coagulant in dairy technology and scientific information about fig latex applications on cheesemaking is very limited. Veringa (1961) reviewed studies using vegetable coagulants and rennet and observed that, after 8 months ripening, Cheddar cheese made with *Ficus carica* extract showed no difference from a control cheese made with animal rennet. Some authors (Rifaat *et al*. 1970; El-Shibiny *et al*. 1973a, b; Oner and Akar, 1993) analysed the use of purified ficin in cheese making and organoleptic tests, demonstrating that no definite differences existed in taste, flavor or body texture. Nouani *et al*. (2009) examined the aptitudes of milk coagulation, stability during conservation and the physic-chemical properties of purified coagulases extracted from the fig tree latex. Although, the use of ficin in traditional cheese production presents a certain reticence since scarces studies exist (Nouani *et al*., 2009); in the Eastern part of Turkey, ficin is used in a milk product called teleme. Teleme has a yogurt like texture with sweet taste and is traditionally prepared by adding a few drops of ficin to milk. Akar and Fadýloğlu (1999) compared purified and unpurified ficin in teleme production. Fadýloğlu (2001) related the properties of free and immobilized ficin (optimum temperature and pH, effect of enzyme and substrate concentration) and their chemical and sensory properties on teleme cheese. Even in Italy there is the tradition to use caprifig latex (*Ficus carica sylvestris*) for producing Cacioricotta (Italian National Research Council, 1996). Faccia *et al*. (2012) employed in their research caprifig latex and calf rennet, respectively, for manufacturing Cacioricotta and comparing their profile of proteolysis. The results of this study showed that caprifig latex exerted a strong proteolyitic activity. Other authors (Scott Blair and Burnett, 1963) reported that the curd made with ficin was only slightly softer and of slightly less weight than curds made with

rennet. Moreover, the use of ficin for milk clotting in the preparation of cheese has been showed to lead to greater bitterness in young cheese. Further hydrolysis of the bitter peptides leads to the disappearance of bitterness on prolonged aging of cheese (Krishnaswami and Johar, 1961).

1.3.5 OTHER COAGULANTS

Other many proteolytic preparations from plants have also been identified as vegetable rennets. *Carica papaya* is a tropical plant belonging to the family of *Caricaceae*. The papaya plant contains a milky latex which is source of proteolytic enzymes: papain and chimopapain. This sap, when dehydrated, is called crude papain. Although all parts of the plant contain latex, only the green, immature fruits are used for latex extraction, because exudations from unripe fruits are much vigorous than from any other part of the plant (Becker, 1958). The use of *Carica papaya* has also been used for long time, even though flavour defects, such as bitterness, have been reported (Veringa, 1961). Nevertheless, despite this drawback, an experiment conducted by Adentunji and Salawu (2008) showed that *Carica papaya* processed cheese was a good supplement for Fe and Mn in food deficient of minerals. *Bromeliaceae* is another plant family whose members usually produce large amounts of peptidases. To date, a number of proteinases from species belonging to this family have been isolated and characterized: stem and fruit bromelain, ananain and comosain, obtained from *Ananas comosus* (Lee *et al*., 1997; Murachi, 1976; Napper *et al*., 1994; Ota *et al*., 1985; Rowan and Buttle, 1994; Rowan *et al*., 1988, 1990). Many studies (Bruno *et al*., 2002, 2010) have been conducted on the purification and biochemical characterization of these enzymes, as well as in milk clotting experiments. However, there is a little information on cheeses obtained with these extracts, due to their excessive proteolytic activity, which affects the sensory profile and the final yield. *Calotropis procera* is another plant, whose crude latex has been used to manufacture *Wara*, a traditional cheese of Nigeria and the Republic of Benin. Dubey and Jagannadham (2003) purified a proteinase from the latex of this medicinal plant. The enzyme named procerain belongs to the cysteine proteinases, like papain and ficin. Even the *Albizia julibrissin*, an ornamental plant of the family of Mimosaceae, is known for its clotting properties. Otani *et al*. (1991) purified a cysteine proteinase from its seeds and they demonstrated that the cheese made with the enzyme was comparable to that obtained with chymosin. In addition, this proteinase did not develop any bitterness in the cheese after three months of ripening. Further studies have been identified other plants sources of for milk-clotting enzymes: *Opuntia phylloclades, Cereus triangularis, Euphorbia caducifolia, E. hista*

(Umar Dahot *et al*., 1990), *Lactuca sativa* (Lo Piero *et al*., 2002), seven papilionoideae species (*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*, *E. psoraleoides*, *Adenolichos anchietae* e *Droogmansia megalantha*; Lopes *et al*., 1998) and *Helianthus annuus* (Park *et al*., 2000).

1.3.6 ENZYMATIC COMPOSITION

In recent decades, interest in plant natural products has grown rapidly. The number of industrially employed enzymes of plant origin is still small but growing fast. In this respect, proteinases are the most commercially important enzymes due to their multiple applications in the food, pharmaceutical and detergent industries, as well as in the preparation of leather and wool (Doran, 2002). One of the most important applications of proteinases in the food industry is the use of rennet in cheese making. Milk-clotting enzymes have been found in almost all kinds of plant tissues and it appears to be a general rule that all proteolytic enzymes possess the ability to clot milk under proper conditions (Tamer and Mavituna, 1997). All enzymes employed for cheese belong to the family of aspartic proteinases (APs), with acidic optima pH and two aspartic acid residues in the catalytic site (Szecsi, 1992; Foltmann, 1993; Chitpinityol and Crabbe, 1998). These two aspartic residues, which are responsible for the catalytic activity, preferentially cleave peptide bonds between residues with hydrophobic side-chains (Domingos et al., 2000). The most widely used AP is rennet, which has chymosin as its active component (Vioque et al., 2000). Some plant APs have shown to possess similar characteristics to calf-derived rennet and hence have attracted attention in cheese industry. As previously described, *Cynara cardunculus* (cardoon) produces cardosin A and cardosin B, which have been shown to share specificity and kinetic parameters with chymosin and pepsin (Verissimo *et al*., 1995, 1996). Therefore, it appears that the enzyme composition of cardoon coagulant is similar to that of calf rennet. However, the presence of APs with milk-clotting activity has been reported in a variety of plant species belonging to the family Asteraceae, i.e., artichoke (*Cynara scolymus* L.), milk thistle (*Silybum marianum* (L.) Gaertn.) (Vairo-Cavalli *et al*., 2005), *Onopordum turcicum* (Tamer, 1993), a thistle related to cardoon (*Centaurea calcitrapa*), (Domingos *et al*., 2000). Although APs are the most employed enzymes for cheese-making, there are some other plant enzymes that belong to the the cysteine proteinases (CPs) family. These proteinases have in their catalytic mechanism a cysteine group in the active site and can also be take into account for dairy industry. Ficin, papain and related plant proteinases such as chymopapain and bromelain belong to this family of enzymes (Turk *et al*., 1997). As mentioned before, ficins isolated from the latex of different *Ficus* trees possess

certain characteristic properties, such as the capacity to digest casein, suggestive of a milk-clotting property (Devaraj *et al*., 2008).

Finally, the progress towards the commercial-scale use of these plant proteinases can be useful for dairy industry. Therefore, further and deeper studies on plant proteinases and their families are suggested.

1.3.7 IMPACT ON CHEESE YIELD, TEXTURAL AND SENSORY PROPERTIES

Cheese yield is expressed as the quantity of cheese of a given dry matter produced from a given quantity of milk with a defined protein and fat content (kg/100kg milk, Fox *et al*., 2000). However, the yield of cheese can vary depending on several factors, i.e., milk composition (fat and casein), species (e.g. cow, goat or sheep), breed, stage of lactation and animal health. Cheese yield is also influenced by technological interventions, i.e., standardization, low concentration factor ultrafiltration and pasteurization as well as rennet type. In this regard, the influence of rennet type on cheese yield is due to its specific and general proteolytic activity. The specific proteolytic activity is also known as clotting activity, which is the ability of the rennet to hydrolyze only the Phe_{105}-Met_{106} bond of κ-casein. The general proteolytic activity is defined as the overall proteolyitc action of the rennet on milk proteins. A high ratio of clotting activity to proteolytic one is an essential requirement for calf rennet substitutes. Therefore, the various rennets used in cheesemaking differ in relation to this ratio. For instance, calf chymosin is the least proteolytic of the gastric protinases, the proteolytic activity of which decreases in the following general order: chicken pepsin > porcine pepsin > ovine pepsin > bovine pepsin > calf rennet (chymosin) ≈ fermentation-produced chymosin. Microbial rennet are also more proteolytic than calf chymosin, with proteolytic activity being in the following order: *Endothia parasitica* > *Mucor miehei* > *Mucor pusillus* > calf chymosin (Fox *et al*., 2000). Plant coagulants are known to possess a high proteolytic activity and for this reason they are not employed for commercial cheesemaking. An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers which have been used for centuries in traditional manufacture of sheep milk cheese in several areas of Portugal and Spain (Macedo *et al*., 1993). Therefore, it is a special feature of cheeses processed with plant coagulants that proteolysis is more pronounced (Chen *et al*., 2003; Prados *et al*., 2007; Galan *et al*., 2008; Pereira *et al*., 2008; Pino *et al*., 2009). This enhanced proteolytic activity is responsible for a lower cheese yield, but also affect negatively the sensory quality of cheese by producing bitterness. Bitterness results from the accumulation of bitter-tasting peptides released by the action of proteolytic

enzymes on caseins (Sullivan and Jago, 1972; Creamer, 1978; Stadhouders *et al*., 1983), which release peptides containing hydrophobic amino acid residues. Ney (1971) reported that peptides with hydrophobicity (Q) values > 1400 cal per mole and molecular masses < 6 kDa display bitterness. Therefore, medium sized (tri- to hexa-) peptides (Biede and Hammond, 1979) and peptides < 6 kDa having a high content of Leu, Pro, Phe, Tyr, Ile and Trp residues are likely to be bitter (FitzGerald and O'Cuinn, 2006). Matoba and Hata (1972) subsequently reported that the presence of internally sited hydrophobic amino acid residues led to greater bitterness than when the hydrophobic residues were located at either the N- or C-terminus in peptides. The presence of internally sited Pro residues was shown to be a major and distinct contributor to peptide bitterness due to the unique conformation associated with this imino acid (Ishibashi *et al*., 1988). The production of bitter peptides during the process of enzymatic digestion is not always unfavourable; indeed, a bitter taste is one of the important components of cheese taste quality (Shinoda *et al*., 1985; 1986). However, when bitter peptides accumulate, their concentration may exceed the flavour threshold for bitterness and can limit acceptance of the cheese (Visser *et al*., 1983a, b). In this regard, the majority of plant rennets have been found to be unsuitable because they produce extremely bitter cheeses due their excessively proteolytic activity. On the contrary, the aqueous extracts of cardoons (*Cynara cardunculus* L.) are an exception to this general behaviour of plant coagulants (Pires *et al*., 1994; Trujillo *et al*., 1994; Vieira de Sá and Barbosa, 1972). The high proteolytic activity of plant proteinases also affect textural properties, leading to accelerated softening on the cheese body as well as liquefaction and shape loss. Chesees made with plant rennets, in fact, are softer and creamier compared with calf rennet and microbial clotting enzymes (Chen *et al*., 2003). A possible way to avoid this excessive proteolysis and contemporary reduce bitterness and weakness of cheese body, is to use milk-clotting enzymes in immobilized systems. The major advantage of immobilization is that the enzyme is "fixed" in order to retain it in a continuous process (Garg and Johri, 1994). Fadyloglu (2001) used immobilized ficin in teleme production, which gave better results in terms of sensory properties in comparison with teleme made by free enzyme. However, further studies on immobilization of plant coagulants need to be carried out in order to enhance current knowledge of it. Although cheeses made with vegetable coagulants can present quality defects (bitter taste and loss of consistency), they are normally produced on an artisanal scale, in a farmhouse or small dairy. Therefore, they have an important socio-economical contribution to the dairy sector at local and regional areas of each country and thus play an important part in the local agricultural economy (Roseiro *et al*., 2003).

1.3.8 RELIGIOUS AND ETHICAL INFLUENCE

At present, particular attention has been directing toward natural rennet extracted from plants for several reasons. As mentioned before, one of these reasons is related to the contribution of plant coagulants in improving the nutritional intake of people whose use of animal rennets is restricted (Gupta and Eskin, 1977). In this regard, plant proteinases can meet the needs of those people who avoid consuming animal-derived products due to diet (vegan and/or vegetarians) and/or religious reasons (e.g., Judaism and Islamism). Both Judaism and Islamism have guidelines about food preparation and diet set forth in their holy books. According to the Jews, in fact, a food is *Kosher*, or acceptable to eat, when prepared in accordance with the conditions set by Judaic law. In this regard, dairy products must come from animals that are fit to be Kosher. Just as Kosher is used in Judaism, *Halal*, when used in relationship to food or drinks, means “permissible for consumption by Muslim”. Any food that the potential to or actually causes a bad effect on the mind, body or spirit is “haram”: "unauthorized or illicit." Enzymes such as rennet used in the coagulation of cheese must be the product of Halal animal slaughtered in the zabihah manner, while enzymes from plants are acceptable (Eliasi and Dwyer, 2002). Therefore, coagulants extracted from plants can find the agreements of particular markets, encouraging contemporaneously scientific investigations on this topic.

2. SCIENTIFIC OBJECTIVES OF THE THESIS

The present work aims to enhance current knowledge of plant coagulants, whose interest is increasing because of the need of innovation in cheese productions and the recent food trends. *Cynara cardunculus* L. and *Ficus carica* L., two well known plant coagulants largely distributed in Mediterrean countries, were at first investigated in their lipolytic activity. The lipolytic activity was characterized by agar plate and chromogenic assays as well as zymogram analysis. The final aim of this step was to assess wheter these plant extracts were sources of lipases, which could give a potential contribution in developing flavour during cheese ripening. The second goal was to evaluate the proteolytic activity of these two plant coagulants. After a preliminary proteinase assay with azocasein, the hydrolysis of bovine milk α_s and β-caseins was studied by eletrophoretic gels in order to assess the contribution of each plant enzyme to the overall proteolytic specificity. The degradation pattern of each bovine casein was also examined quantitatively by densitometric analysis. Finally, both these plant proteinases were investigated for their ability to produce antioxidant peptides/hydrolysates from bovine milk casein (sodium caseinate and β-casein). Therefore,

this work intends to promote the use of *Cynara cardunculus* L. and *Ficus carica* L. as potential plant rennets in cheese production as sources of lipases, proteinases and bioactive peptides. In this regard, plant coagulants can contribute to the growing commercial interest in the context of natural and health-promoting functional foods, which can meet the needs of specific target of consumers, i.e., vegetarians and/or vegans. In future, the detailed understanding of plant coagulants will gradually increase and it is expected that the development and the use of these new milk-clotting enzymes will continue.

3. REFERENCES

Adetunji, V. O. and Salawu, O. T. (2008) 'West african soft cheese "wara" processed with *Calotropis procera* and *Carica papaya*: a comparative assessment of nutritional values', *African Journal of Biotechnology*, 7 (18), 3360-3362.

Agudelo, R. A., Gauthier, S. F., Pouliot, Y., Marin, H. and Savoie, L. (2004) 'Kinetics of peptide fraction release during in vitro digestion of casein', *Journal of the Science of Food and Agriculture*, 84, 325-333.

Akar, B. and Fadýloğlu, S. (1999) 'Teleme production by purified ficin', *Journal of Food Quality*, 22, 671-680.

Andrén, A. (1982) *Chymosin and Pepsin in Bovine Abomasal Mucosa Studied by Use of Immunological Methods*, PhD Thesis, Department of Animal Husbandry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Andrén, A. (1998) 'Milk-clotting activity of various rennets and coagulants: background and information regarding IDF standards', *International Dairy Federation, Commission B, on the Use of Enzymes in Dairying*, Reykjavik, Iceland, 28 Aug 1997, Brussels: Document No. 332, 9-14.

Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J. and Baeyens-Volant, D. (2011) 'Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from *Ficus carica* latex', *Phytochemistry*, 72, 1718-1731.

Awad, S., Lüthi-Peng, Q. Q. and Puhan, Z. (1998) 'Proteolytic activities of chymosin and porcine pepsin on buffalo, cow, and goat whole and beta-casein fractions', *Journal of Agricultural and Food Chemistry*,46, 4998-5007.

Awad, S., Lüthi-Peng, Q. Q. and Puhan, Z. (1999) 'Proteolytic activities of Suparen and Rennilase on buffalo, cow, and goat whole casein and β-casein', *Journal of Agricultural and Food Chemistry*, 47, 3632-3639.

Becker, S. (1958) 'The production of papain- an agricultural industry for tropical America', *Economy Botany*, 12(1), 62-79.

Biede, S. L. and Hammond, E. G. (1979) 'Swiss cheese f1avor. II. Organoleptic analysis', *Journal of Dairy Science*, 62, 238-248.

Broome, M. C., Xu, X. and Mayes, J. J. (2006) 'Proteolysis in Cheddar cheese made with alternative coagulants', *Australian Journal of Dairy Technology*, 61, 85-87.

Bruno, M. A., Pardo, M. F., Caffini, N. O. and López, L. M. I. (2002) 'Purification of a New Endopeptidase Isolated from Fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*)', *Acta Farmacéutica Bonaerense*, 21 (1), 51-56.

Bruno, M. A., Lazza, C. M., Errasti, M. E., López, L. M. I., Caffin, N. O. and Pardo, M. F. (2010) 'Milk clotting and proteolytic activity of an enzyme preparation from *Bromelia hieronymi* fruits', *LWT - Food Science and Technology*, 43, 695-701.

Cattaneo, T. M. P., Nigro, F., Messina, G. and Giangiacomo, R. (1994) 'Effect of an enzymatic complex from pineapple pulp on the primary clotting phase', *Milchwissenschaft*, 49, 269-272.

Cavalcanti, M. T. H., Teixeira, M. F. S., Lima Filho, J. L. and Porto A. L. F. (2004) 'Partial purification of new milk-clotting enzyme produced by *Nocardiopsis sp*', *Bioresource Technology*, 93, 29-35.

Chazarra, S., Sidrach, L., Lopez-Molina, D. and Rodriguez-Lopez, J. (2007) 'Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus*, L.) flowers', *International Dairy Journal*, 17, 1393-1400.

Chen, S. J, Agboola, S. and Zhao, J. (2003) 'Use of Australian cardoon extract in the manufacture of ovine milk cheese, a comparison with commercial rennet preparations', *International Journal of Food Science and Technology*, 38, 799‑807.

Chitpinityol, S. and Crabbe, M. J. C. (1998) 'Chymosin and aspartic proteinases', *Food Chemistry*, 61, 395-418.

Cordeiro, M. C., Jakob, E., Puhan, Z., Pais, M. S. S. and Brodelius, P. E. (1992) 'Milk clotting and proteolytic activities of purified cynarases from *Cynara cardunculus* - a comparison to chymosin', *Milchwissenschaft*, 47, 681-748.

Cordeiro, M. C., Pais, M. S. and Brodelius, P. E. (1994) 'Tissue-specific expression of multiple forms of cyprosin (aspartic proteinase) in flowers of *Cynara cardunculus*', *Physiologia Plantarum*, 92, 645-653.

Creamer, L. K. (1978) 'Degradation of casein components during cheese ripening', In: *Fifth International Gongress of Food Science and Technology*, Kyoto, Japan (abstr), 57.

Deschamps, J. P. (1840) 'De la pressure', *Journal of Pharmacology*, 26, 412-420.

Dewane, R. A., (1960) 'Rennet substitutes', *A Review of Literature*, Paul Lewis Laboratories, Inc, Milwaukee, Wis.

Domingos, A., Cardos, P. C., Xue, Z-T, Clemente, A., Brodelius, P. E. and Pais, M. S. (2000) 'Purification, cloning and autoproteolytic processing of an aspartic proteinase from *Centaurea calcitrapa*', *European Journal of Biochemistry*, 267, 6824-6831.

Doran, P. M. (2002) 'Properties and applications of hairy-root cultures', in Oksman-Caldenty K-M, BarzWH, eds., *Plant biotechnology and transgenic plants*, New York: Mercel Dekker Inc., 143-162.

Duarte, A. R., Duarte, M. D. R., Moreira, K. A., Cavalcanti, M. T. H., de Lima, J. L. and Porto, A. L. F. (2009) '*Jacaration corumbensis O. Kuntze* a new vegetable source for milk-clotting enzymes' *Brazilian Archives of Biology and Technology*, 52, 1-9.

Dubey, V. K. and Jagannadham, M.V. (2003) 'Procerain, a stable cysteine protease from the latex of *Calotropis procera*', *Phytochemistry*, 62, 1057-1071.

Eliasi, J. R. and Dwyer, J. T. (2002) 'Kosher and Halal: religious observances affecting dietary intakes', *Journal of the American Dietetic Association*, 101(7), 911-913.

El-Shibiny, S., Rifaat, I. D., Fahmi, A. H. and Abd El-Salam, M. H. (1973a) 'Studies of milk clotting enzymes from plant sources. III. The manufacture of Domiati cheese with the prepared enzyme from fig latex var. Soltani', *Egyptian Journal of Food Science*, 1(2), 235-240.

El-Shibiny, S., Abd El-Salam, M. H., Rifaat, I. D. and Fahmi, A. H. (1973b) 'Studies on milk clotting enzymes from plant sources. II. Separation of milk clotting enzymes from *Ficus carica* var. Soltani', *Sudan Journal of Food Science and Technology*, 5, 30-34.

Egito, A. S., Girardet, J. M., Laguna, L. E., Poirson, C., Mollé, D., Miclo, L., Humbert, G. and Gaillard, J. L. (2007) 'Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k-casein', *International Dairy Journal*, 17, 816-825.

Esteves, C. (1995) '*Estudo comparativo das características bioquímicas dos coalhos de Cynara cardunculus L., Cynara scolymus L., e Cynara humilis L.*', M.Sc. thesis, Universidade de Coimbra, Portugal.

Faccia, M., Picariello, G., Trani, A., Loizzo, P., Gambacorta, G., Lamacchia, C. and Di Luccia, A. (2012) 'Proteolysis of Cacioricotta cheese made from goat milk coagulated with caprifig (*Ficus carica sylvestris*) or calf rennet', *European Food Research and Technology*, 234, 527-533.

Fadyloglu, S. (2001) 'Immobilization and characterization of ficin', *Nahrung / Food*, 45, 143-146.

Faro, C. J. (1991) '*Purificação e caracterização fisico-química da protease de Cynara cardunculus L.*', PhD thesis, Universidade de Coimbra, Portugal.

Faro, C. J., Moir, A. J. and Pires, E. V. (1992) 'Specificity of a milk-clotting enzyme extracted from the thistle *Cynara cardunculus* L.: action on oxidized insulin and κ-casein', *Biotechnology Letters*, 14, 841- 846.

Faro, C. J., Veríssimo, P., Lin, Y., Tang, J. and Pires, E. V. (1995) 'Cardosin A, an abundant aspartic protease, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L.', in Takahashi, K. (ed.), *Aspartic proteinases: Structure, Function, Biology and Biomedical Implications*, New York, USA: Plenum Press, 373-377.

Fernández, J., Curt, M. D. and Aguado, P. L. (2006) 'Industrial applications of *Cynara cardunculus* L. for energy and other uses', *Industrial Crops and Products*, 24, 222-229.

FitzGerald, R. J. and O'Cuinn, G. (2006) 'Enzymatic debittering of food protein hydrolysates', *Biotechnology Advances*, 24, 234-237.

Flamm, E. L. (1991) 'How FDA approved chymosin: a case history', *Biotechnology*, 9, 349-351.

Foltmann, B. F. (1966) 'A review on prorennin and rennin', *Comptes Rendus des Travaux des Laboratoire Carlsberg*, 35(8), 143-231.

Foltmann, B. F. (1992) 'Chymosin: a short review on foetal and neonatal gastric proteases', *Scandinavian Journal of Clinical and Laboratory Investigations*, 52 (Suppl 210), 65-79.

Foltmann, B. F. (1993) 'General and molecular aspects of rennets', in Fox, P. F., ed., *Cheese: Chemistry, Physics and Microbiology, Volume 1: General Aspects*, London: Chapman & Hall, 37-68.

Fox, P. F, Guinee, T. P, Cogan, T. M and McSweeney, P. L. H. (2000) 'Cheese yield', in Springer, ed., *Fundamentals of Cheese Science*, Gaithersburg Maryland: Aspen Publishers, Inc., 169-205.

Fox, P. F. and McSweeney, P. L. H. (2004) 'Cheese: an overview', in Fox, P. F., McSweeney, P. L. H., Cogan, T. M. and Guinee T. P., eds., *Cheese: Chemistry, Physics and Microbiology, Volume 1: General Aspects*, 3rd ed., London, UK: Elsevier Academic Press, 1-18.

Galan, E., Prados, F., Pino, A., Tejada, L. and Fernández-Salguero, J. (2008) 'Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheeses made with sheep milk', *International Dairy Journal*, 18, 83-98.

Garg, S. K. and Johri, B. N. (1994) 'Rennet: current trends and future research', *Food Reviews International*, 10, 313-355.

Gorini, C. (1892), *Rivista di Igiene e Sanità pubblica*, 3, 527.

Gorini, C. (1893), *Hygienische Rundschau*, 3, 381.

Gorini, C., (1930) 'Rennin production by *Bacillus prodigiosus*', *Journal of Bacteriology*, 20, 297-298.

Green, M. L. (1972) 'Assessment of swine, bovine and chicken pepsin as rennet substitutes for cheddar cheese-making', *Journal of Dairy Research*, 39, 261-265.

Guinee, T. P. and Wilkinson, M. G. (1992) 'Rennet coagulation and coagulants in cheese manufacture', *Journal of the Society of Dairy Technology*, 45, 94-104.

Gupta, C. B. and Eskin, N. A. (1977) 'Potential use of vegetable rennet in the production of cheese', *Food Technology*, 5, 62-66.

Harboe, M. K. (1992a) 'Coagulants of different origin', *European Dairy Magazine*, 2, 6-15.

Harboe, M. K. (1992b) 'Chymogen, a chymosin rennet manufactured by fermentation of *Aspergillus niger*. *Fermentation-Produced Enzymes and Accelerated Ripening in Cheesemaking*', Document No. 269, International Dairy Federation, Brussels, 3-7.

Harboe, M. K. (1993) 'Chymogen, Chr. Hansen's fermentation produced rennet', in Mathieu, J., ed., *Milk Proteins '93*, La Roche-sur Foron, France: ENILV, 122-131.

Harboe, M., Broe, M. L and Qvist, K. B. (2010) 'The Production, Action and Application of Rennet and Coagulants', in Law, B. A. and Tamime, A. Y., eds., *Technology of cheesemaking*, 2nd ed., New Delhi: Wiley Blackwell, 98-129.

Heimgartner, U., Pietrzak, M., Geertsen, R., Brodelius, P., Silva-Figueiredo, A. C. and Pais M. S. S. (1990) 'Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*', *Phytochemistry*, 29, 1405-1410.

Ibiama, E. and Griffiths, M. W. (1987) 'Studies on a milk coagulating enzyme, 'Calotropain', obtained from Sodom apple (*Calotropis porcera*)', *Journal of Food Agriculture*, 1, 157-162.

Ishibashi, N., Ono, I., Kato, K., Shigenaga, T., Shinoda, H., Okai, H. and Fukui, S. (1988) 'Role of the hydrophobic amino acid residue in the bitterness of peptides', *Agricultural and Biological Chemistry*, 52(1), 91-94.

Italian National Research Council (1996) 'I prodotti caseari del Mezzogiorno', In: Tecnos SRL, ed., vol II. Milan.

IUBMB, (1992) 'Recommendations of the nomenclature committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes', Enzyme Nomenclature, New York: Academic Press Inc., 403-408.

Jacob, M., Jaros, D. and Rohm, H. (2011) 'Recent advances in milk clotting enzymes', *International Journal of Dairy Technology*, 64(1), 14-33.

Krishnaswami, M. A. and Johar, D. S. (1961) 'Preparation of cheddar and processed cheese using the milk clotting enzyme of *Ficus curica*', *Proeedings of the Symposium on Proteins*, Mysore, India, 1960, Chem. Res. Comm. SOC. Bipl. Chemists: 302-306.

Lansky, E. P., Paavilainen, H. M., Pawlus, A. D. and Newman, R. A. (2008) '*Ficus* spp. (fig): Ethnobotany and potential as anticancer and anti-inflammatory agents', *Journal of Ethnopharmacology*, 119, 195-213.

Lee, K. L., Albee, K. L., Bernasconi, R. J. and Edmunds, T. (1997) 'Complete amino acid sequence of ananain and a comparison with stem bromelain and other plant cysteine proteases', *Biochemical Journal*, 327, 199-202.

Libouga, D. G., Vercaigne-Marko, D. and Djangal, S. L. (2006) 'Study of a suitable cheese making milk clotting agent from Balanites aegyptica fruits', *Tropicultura*, 24, 229-238.

Llorente, B. E., Brutti, C. B. and Caffini, N. O. (2004) 'Purification and characterization of a milk-clotting aspartic proteinase from globe artichoke (*Cynara scolymus*, L.)', *Journal of Agricultural and Food Chemistry*, 52, 8182-8198.

Lo Piero, A. R., Puglisi, I. and Petrone, G. (2002*)* 'Characterization of 'Lettucine,'' a serine-like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting', *Journal of Agriculture and Food Chemistry*, 50, 2439-2443.

Lopes, A., Teixeira, G., Liberato, M. C., Pais, M. S. S. and Clemente, A. (1998) 'New vegetal source for milk clotting enzymes', *Journal of Molecular Catalysis B: Enzymatic*, 5, 63-68.

Low, Y. H., Agboola, S., Zhao, H. and Lim, M. Y. (2006) 'Clotting and proteolytic properties of plant coagulants in regular and ultrafiltered bovine skim milk', *International Dairy Journal*, 16, 335-343.

Macedo, I. (1993) 'Especificidade caseinolítica da protease de *Cynara cardunculus* L.', Ph.D. thesis, Universidade de Coimbra, Portugal.

Macedo, I., Faro, C. J. and Pires, E. M. (1993) 'Specificity and kinetics of the milk-clotting enzyme from cardoon (*Cynara cardunculus* L.) toward bovine κ-casein', *Journal of Agricultural and Food Chemistry*, 41**,** 1537-1547.

Malenotti I., (1832) 'Il manuale del pecoraio', Colle [Val d'Elsa], Tipografia Pacini E., 1-215.

Matoba, T. and Hata, T. (1972) 'Relationship between bitterness of peptides and their chemical structure', *Agricultural and Biological Chemistry*, 36(8), 1423- 1431.

Moharib, S. A. (2004) 'Protease activities in seeds of fenugreek (*Trigonella foenum-graecum*)', *Advances in Food Sciences*, 26, 69-74.

Murachi, T. (1976) 'Bromelain enzymes', *Methods in Enzymology*, 45, 475-485.

Napper, A. D., Bennett S. P., Borowski M., Holdridge M. B., Leonard M. J. C., Rogers E. E., Duan Y., Laursen R. A., Reinhold, B. and Shames, S. L. (1994) 'Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain', *Biochemical Journal*, 301, 727-735.

Ney, K. H. (1971) 'Prediction of bitterness of peptides from their amino acid composition', *Z Lebensm-Unters Forsch*, 147, 64-68.

Nouani, A., Dako, E., Morsli, A., Belhamiche, N., Belbraouet, S. , Bellal, M.M. and Dadie, A. (2009) 'Characterization of the purified coagulant extracts derived from artichoke flowers (*Cynara scolymus*) and from the fig tree latex (*Ficus carica*) in light of their use in the manufacture of traditional cheeses in Algeria', *Journal of Food Technology*, 7(1), 20-29.

Oner, D. M. and Akar, B. (1993) 'Separation of the proteolytic enzymes from fig tree latex and its utilization in Gaziantep cheese production', *Lebensmittel Wissenschaft und Technologie*, 26, 318-321.

Orosin, B., Veselov, I. Ya, Smirnova, T. A. and Ivanova, T. V. (1970) 'Effect of culture conditions on the biosynthesis of milk-clotting and proteolytic enzymes by Bacillus mesentericus strain 11-11', *Prikladnaya Biokhimiya i Mikrobiologiya*, 6 (6), 660-665.

Ota, S., Muta E., Katahira, J. and Okamoto, Y. (1985) 'Reinvestigation of fractionation and some properties of the proteolytically active components of stem and fruit bromelains' *Journal of Biochemistry*, 98, 219-228.

Otani, H., Matsumori, M. and Hosono A., (1991) 'Purification and some properties of a milk clotting protease from the young seeds of *Albizia julibrissin*', *Animal Science and Technology*, 62, 424-432.

Padhmanabhan, C., Chitre, A. and Sastri, N. V. (1993***)*** 'Milk clotting proteinase isolated from *Dieffenbachia maculata*', *Nahrung/Food*, 37, 99-101.

Pais, M. S. S. (2002) 'The cheese those romans already used to eat. from tradition to molecular biology and plant biotechnology', *Memórias da academia das ciências de Lisboa*, 1-17.

Papoff, C. M., Mauriello, R., Pirisi, A., Piredda, G., Addis, M. and Chianese, L. (2004) 'Proteolytic activity of animal rennet on ovine casein', *Michwissenschaft*, 59, 414-417.

Park, H., Yamanaka, N., Mikkonen, A., Kusakabe, I. and Kobayashi, H., (2000) 'Purification and characterization of aspartic proteinase from sunflower seeds', *Bioscience, Biotechnology and Biochemistry*, 64, 931-939.

Patil, S. V., Salunke, B. K. and Bhat, J. A.,(2003) 'Herbal rennet from *Calotropis gigantea*', *Journal of Medicinal and Aromatic Plant Science*, 25, 392-396.

Pereira, C. I., Gomes, E. O., Gomes, A. M. P. and Malcata, F. X. (2008) 'Proteolysis in model Portuguese cheeses: effects of rennet and starter culture', *Food Chemistry*, 108, 862-868.

Pino, A., Prados, F., Galan, E., McSweeney, P. L. H. and Fernández-Salguero, J. (2009) 'Proteolysis during the ripening of goats milk cheese made with plant coagulant or calf rennet', *Food Research International*, 42, 324-330.

Pires, E., Faro, C., Macedo, I., Esteves, C., Morgado, J., Veríssimo, P., Pereira, D. and Gomes, D. (1994) 'Flor de cardo versus quimosina no fabrico de queijos artesanais', *Química*, 54**,** 66-68.

Prados, F., Pino, A. and Fernández-Salguero, J. (2007) 'Effect of a powdered vegetable coagulant from *Cynara cardunculus* in the accelerated ripening of Manchego cheese', *International Journal of Food Science and Technology*', 42, 556-561.

Puhan, Z. and Irvine, D. M. (1973) 'Proteolysis by Proteases of Bacillus subtilis used to make canadian cheddar cheese', *Journal of Dairy Science*, 55 (3), 317-322.

Ramalho-Santos, M., Pissarra, J., Verissimo, P., Pereira, S., Salema, R., Pires, E. and Faro, C. J. (1997) 'Cardosin A, an abundant aspartic protease, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L.', *Planta*, 203**,** 204-212.

Raposo, S. and Domingos, A. (2008) 'Purification and characterization of milk-clotting aspartic proteinases from Centaura calciptrea cell suspension cultures', *Process Biochemistry*, 43, 139-144.

Repelius, C. (1993) 'Application of Maxiren in cheesemaking', in Mathieu, J., ed., *Milk Proteins '93*, La Roche-sur-Foron, France: ENILV, 114-121.

Reps, A., Poznanski, S., Kaszycka, A. and Wasilewski, R. (1974) 'Proteolytic activity of microbial rennet substitutes', *Rocz. Inst. Przem. Mlecz*, 16, 41.

Rifaat, I. D., El-Shibiny, S., El-Salam, M. H. A. and Fahmi, A. H. (1970) 'Studies on milk clotting enzymes from higher plants. I. Milk clotting activity from some local plants', *Indian Journal of Dairy Science*, 23(3), 151-154.

Robbins, B. H. (1930) 'A proteolytic enzyme in ficin, the anthelmintic principle of leche de higueron', *Journal of Biological Chemistry*, 87, 251-257.

Robinson, R. K. and Wilbey, R. A. (1998) 'Cheesemaking Practice', 3rd ed., Gaithersburg, MD: Aspen Publishers, 364-365.

Roseiro, L. B., Barbosa, M., Ames, J. M. and Wilbey, A. (2003) 'Cheesemaking with vegetable coagulants-The use of *Cynara* L. for the production of ovine milk cheese', *International Journal of Dairy Technology*, 56, 76-85.

Rowan, A. D., Buttle, D. J. and Barrett, A. J. (1988) 'Ananain: a novel cysteine proteinase found in pineapple stem', *Archives of Biochemistry and Biophysics*, 267, 262-270.

Rowan, A. D., Buttle, D. J. and Barrett, A. J. (1990) 'The cysteine proteinases of the pineapple plant', *Biochemical Journal*, 266, 869-875.

Rowan, A. D. and Buttle, D. J. (1994) 'Pineapple cysteine endopeptidases', *Methods in Enzymology*, 244, 555-568.

Salhi-Hannachi, A., Chatti, K., Saddoud, O., Mars, M., Rhouma, A.,Marrakchi, M. and Trifi, M. (2006) 'Genetic diversity of different Tunisian fig (*Ficus carica* L.) collections revealed by RAPD fingerprints', *Hereditas*, 143, 15-22.

Salvadori Del Prato, O. (1998) 'Trattato di tecnologia casearia'. Bologna: Edagricole-Calderini, XXVIII -1070.

Sardinas, J. L. (1972) 'Microbial rennets', *Advances in Applied Microbiology*, 15, 39.

Szecsi, P. B. (1992) 'The aspartic proteases', *Scandinavian Journal of Clinical Laboratory Investigations*, 52, 5-22.

Scott Blair, G. W. and Burnett, J. (1963) 'A simple method for detecting an early stage in coagulation of rennetted milk', *Journal of Dairy Research*, 30, 383-390.

Senthilkumar, S., Ramasamy, D. and Subramanian, S. (2006) 'Isolation and partial characterisation of milk-clotting aspartic protease from *Streblus asper*' *Food Science and Technology International*, 12, 103-109.

Shinoda, I., Tada, M., Otagiri, K. and Okai, H. (1985) 'Bitter taste of Pro-Phe-Pro-Gly-Pro-lle-Pro corresponding to the partial sequence (positions 61-67) of bovine β-casein. In: *Peptide Chemistry 1985: 23rd Symposium on Peptide Chemistry (proceedings)*, Kyoto, 287-290.

Shinoda, I., Tada, M., Okai, H. and Fukui, S. (1986) 'Bitter taste of H-Pro-Phe-Pro-Gly-Pro-lle-Pro-OH corresponding to the partial sequence (positions 61-67) of bovine β-casein, and related peptides', *Agricultural and Biological Chemistry*, 50, 1247-1254.

Sgarbieri, V. C., Gupte, S. M., Kramer, D. E. and Whitaker, J. R. (1964) 'Ficus enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* lattices', *The Journal of Biological Chemistry*, 239, 2170-2177.

Sidrach, L., García-Cánovas, F., Tudela, J. and Rodríguez-López, J. N. (2005) 'Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A', *Phytochemistry*, 66, 41-49.

Silva, S. V. and Malcata, F. X. (2000) 'Action of cardosin A from *Cynara humilis* on ovine and caprine caseinates', *Journal of Dairy Research*, 67, 449-457.

Srinivasan, R. A., Anantharamaiah, S. N., Keshavamurthy, N., Anantakrishnan, C. P. and Iya, K. K. (1962) 'Studies on milk clotting bacterial enzyme for cheese making. Part I: Some factors influencing the elaboration of the enzyme by bacteria', *XVIth International Dairy Congress, Copenaghen*, Section IV 1: 401-409.

Stadhouders, J., Hup, G., Exterkate, F. A. and Visser, S. (1983) 'Bitter flavour in cheese. 1. Mechanism of the formation of the bitter flavour defect in cheese', *Netherlands Milk and Dairy Journal*, 37, 157-167.

Sullivan, J. J. and Jago, G. R. (1972) 'The structure of bitter peptides and their formation from casein', *Australian Journal of Dairy Technology*, 27, 98-104.

Tam, J. R. and Whitaker, J. R. (1972) 'Rate and extents of proteolysis of several caseins by pepsin, rennin, *Endiothia parasitica* protease and *Mucor pusillus* protease', *Journal of Dairy Science*, 55, 1523-1533.

Tamer, M. I. (1993) 'Identification and partial purification of a novel milk-clotting enzyme from *Onopordum turcicum*', *Biotechnology Letters*, 13, 427-432.

Tamer, M. I. and Mavituna, F. (1997) 'Protease from freely suspended and immobilized *Mirabilis jalapa*', *Process Biochemistry*, 32, 195-200.

Teixeira, G., Santana, A. R., Pais, M. S. and Clemente, A. (2000) 'Enzymes of *Opuntia ficus-indica* (L.) miller with potential industrial applications', *Applied Biochemistry and Biotechnology*, 88, 299-312.

Trujillo, A. J., Carretero, C. and Guamis, B. (1994) 'Los coagulantes en la industria quesera', *Alimentación, Equipos y Tecnologia*, 13, 91-97.

Trujillo, A. J., Guamis, B., Laencina, J. and Lopez, M. B. (2000) 'Proteolytic activities of some milk clotting enzymes on ovine casein', *Food Chemistry*, 71, 449-457.

Tsouli, J. (1974) 'Etude comparée de l'activité enzymatique de 3 variétés d'artichauts du genre *Cynara cardunculus* L. sur la coagulation du lait', *Le Lait*, 537**,** 415-421.

Tubesha, Z. A. and Al-Delaimy, K. S. (2003) 'Rennin-like milk coagulant enzyme produced by a local isolate of *Mucor*', *International Journal of Dairy Technology*, 56, 237-241.

Turk, B., Turk, V. and Turk, D. (1997) 'Structural and functional aspects of papain-like cysteine proteases and their protein inhibitors', *Biological Chemistry*, 378, 141-150.

Umar Dahot, M., Yakoub Khan, M. and Memon, A. N. (1990) 'Screening of some Pakistani plants for milk clotting activity', *Journal of Islamic Academy of Sciences*, 3, 284-286.

Ustunol, Z. and Zeckzer, T. (1996) 'Relative proteolytic action of milk-clotting enzyme preparations on bovine alpha-, beta- and kappa-casein', *Journal of Food Science*, 61, 1136-1138.

Vairo-Cavalli, S. E., Claver, S., Priolo, N. and Natalucci, C. (2005) 'Extraction and partial characterization of a coagulant preparation from *Silybum marianum* flowers. Its action on bovine caseinate', *Journal of Dairy Research*, 72, 271-275.

Vairo Cavalli, S., Silva, S. V., Cimino, C., Malcata, F. X. and Priolo, N. (2008) 'Hydrolysis of caprine and ovine milk proteins, brought about by aspartic peptidases from *Silybum marianu* flowers', *Food Chemistry*, 106, 997-1003.

Vanderporten, R. and Weckx, M. (1972) 'Breakdown of casein by rennet and microbial milk-clotting enzymes', *Netherlands Milk and Dairy Journal*, 26 47-59.

Veringa, H. A. (1961) 'Rennet substitutes-a review', *Dairy Science Abstracts*, 23, 197-200.

Veríssimo, P., Esteves, C., Faro, C. and Pires, E. (1995) 'The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities', *Biotechnology Letters*, 17, 621-626.

Veríssimo, P., Faro, C. J., Moir, A. J., Lin, Y., Tang, J. and Pires, E. (1996) 'Purification, characterization and partial amino acid sequencing of two new aspartic proteinases from fresh flowers of *Cynara cardunculus* L.', *European Journal of Biochemistry*, 235, 762-768.

Veríssimo, P., Ramalho-Santos, M., Faro, C. and Pires, E. (1998) 'A comparative study on the aspartic proteinases from different species of *Cynara*', *Aspartic Proteinases*, 436, 459-463.

Vieira de Sá, F. and Barbosa, M. (1972) 'Cheese-making with a vegetable rennet from Cardo (*Cynara cardunculus*)', *Journal of Dairy Research*, 39**,** 335-343.

Vioque, M., Gomez, R., Sanchez, E., Mata, C., Tejada, L. and Fernández-Salguero, J. (2000) 'Chemical and microbiological characteristics of ewe's milk cheese manufactured with extracts from flowers of *Cynara cardunculus* and *Cynara humilis* as coagulants', *Journal of Agriculture and Food Chemistry*, 48, 451-456.

Visser, S., Slangen, K. J., Hup, G. and Stadhouders, J. (1983a) 'Bitter f1avourin cheese. 3. Comparative gel-chromatographic analysis of hydrophobic peptide fractions from twelve Gouda type cheeses and identification of bitter peptides isolated from a cheese made with *Streptococcus cremoris* strain HP', *Netherland Milk Dairy Journal*, 37, 181-192.

Visser, S., Slangen, K. J., Hup, G., Exterkate, F. A. and Stadhouders, J. (1983b) 'The bitter f1avour defect in cheese; some chemical and microbiological aspects', *Netherland Milk Dairy Journal*, 37, 250-251.

Wahlin, J. G. (1928) 'A study of rennin action. I. Rennin production by *Bacillus prodigiosus*', *Journal of Bacteriology*, 16, 355-373.

Whitaker, J. R. (1972) *Principles of Enzymology for the Food Sciences*, pp. 30-35, New York: Marcel Dekker.

Wigley, R. C. (1996) 'Cheese and whey', in Godfrey, T. and West, S., eds., *Industrial Enzymology*, 2nd ed., London: Macmillan Press, 133-54.

CHAPTER 2

Characterization of lipolytic and esterolytic activity

ABSTRACT

Esterase/lipase activity of *Cynara cardunculus* L. and *Ficus carica* L. was evaluated. These plant coagulants possessed esterase and not lipase activity with major affinity for acetetate, propionate and butyrate esters. At first, the enzymatic activity was characterized by an agar plate assay. In this assay it was demonstrated that the clear supernatant (CS) and the gummy material (GM) of *Ficus carica* expressed a similar enzymatic activity. Thus only CS was considered for the next biochemical characterization. The temperature and pH profiles were also determined. It was found that *Cynara cardunculus* and *Ficus carica* displayed optimal activity in a range temperature of 40-60°C and neutral-alkaline pHs (7.5-8.0). Finally, the study of these esterases by zymography revealed the presence, in CS, of several isoforms differing in substrate specificity and electrophoretic mobility, indicating a high level of polymorphism.

Keywords: esterase, lipase, *Cynara cardunculus* L., *Ficus carica* L., zymography, 2D

INTRODUCTION

Lipolytic enzymes are widely distributed in animals, plants and microorganisms (Bornscheur, 2002). They can be classified as esterases (E.C. 3.1.1.1, carboxyl ester hydrolases) or lipases (E.C. 3.1.1.3, triacylglycerol hydrolases). Esterases catalyse the cleavage of ester bonds of short chain length fatty acids, while true lipases have marked preference for long chain fatty acid substrates (Jeager *et al.*, 1999). These enzymes are mostly used in the processing of fats and oils, detergents, production of cosmetics, pharmaceuticals and food industry (Kazlauskas and Bornscheuer, 1998). In dairy industry, lipases are involved in the development of flavour by the catabolism of fatty acids known as lipolysis. Lipolysis is an important biochemical event occurring during cheese ripening, which releases free fatty acids (FFA) that contribute directly to cheese aroma. In this regard, long-chain FFA (> 12

carbon atoms) are considered to play a minor role in cheese flavour due to their high perception thresholds. Short and intermediate-chain, even-numbered fatty acids ($C_{4:0}$–$C_{12:0}$) have considerably lower perception thresholds and each gives a characteristic flavour note (Molimard and Spinnler, 1996). Lipases originate in cheese from different sources such as the type of rennet. It has been showed that commercial rennets are normally free from lipolytic activity. However rennet paste, used in some kind of hard Italian cheeses (e.g., Provolone, Romano), contains a potent lipase, pregastric esterase (PGE), which is responsible for the extensive lypolysis and in the characteristic "piccante" flavour of such varieties (Fox *et al*., 2000). In this respect, lipases from plant coaugulants could be also considered as potential source of flavour. Despite the fact that plant rennets and their milk-clotting enzymes have been well documented and studied (Cattaneo *et al.*, 1994; Teixeira *et al.*, 2000; Fadyloglu, 2001; Llorente *et al.*, 2004; Chazarra *et al.*, 2007; Egito *et al*., 2007; Raposo and Domingos, 2008; Vairo Cavalli *et al.* 2008; Duarte *et al.*, 2009), little information (Sousa and Malcata, 1997) is available on their esterases/lipases and their potential contribution to cheese flavour. To our knowledge, no previous investigations report on the biochemical characterization of lipases/esterases belonging to *Cynara cardunculus* L. extract and *Ficus carica* L. latex. *Cynara cardunculus* L. is a perennial herb native to the Mediterranean region and its flowers extract has been used for years in the manufacture of several traditional Portuguese and Spanish cheeses (Silva *et al.*, 2002); while *Ficus carica* L. latex, a fruit crop species well suited for the environmental conditions of the Mediterranean countries (Salhi et-Hannachi *et al.*, 2006), has been employed in a milk product that is called teleme in the Eastern part of Turkey (Fadyloglu, 2001).

In the present chapter it is given an overview of milk lipids, the main important agents of lypolysis in cheese and their contribution to flavour. Thus, the work aims to characterize esterases/lipases of *Cynara cardunculus* and *Ficus carica* in some of their biochemical properties, i.e., substrate specificity, optimal pH and temperature, which might be helpful for a possible industrial application such as cheesemaking.

1. MILK LIPIDS

Bovine milk typically contains, ca. 3.5-5 g fat $100mL^{-1}$ in the form of emulsified globules ranging from 0.1 to 10 mm in diameter (McPherson and Kitchen, 1983; Jensen *et al*., 1991). The main lipids of milk are triacylglycerides, which may represent up to 98% of the total lipids (Christie, 1983; Jensen *et al*., 1991; Gunstone *et al*., 1994). The remaining 1-2 g

100 g^{-1} consists of phospholipids (0.8 g 100 g^{-1}), diacylglycerols, sterols (0.3 g 100 g^{-1}) and trace quantities of carotenoids, fat-soluble vitamins and traces of free fatty acids (FFA) (Jensen, 2002; Huppertz *et al*., 2009). The fat in milk exists in the form of dispersed globules, surrounded by a lipoprotein membrane called the milk fat globule membrane (MFGM) (Keenan and Maher, 2006). Ruminant milk fats contain a wide range of fatty acids and 437 distinct acids have been identified in bovine milk fats. The major free fatty acids (FFA) found in milk fat are butanoic ($C_{4:0}$), hexanoic ($C_{6:0}$), octanoic ($C_{8:0}$), decanoic ($C_{10:0}$), dodecanoic ($C_{12:0}$), tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), octadecanoic ($C_{18:0}$), cis-9-octadecenoic ($C_{18:1}$), cis, cis-9,12-octadecadienoic ($C_{18:2}$), and 9,12,15-octadecatrienoic acids ($C_{18:3}$) (Jensen *et al*., 1962; Banks, 1991; Jensen *et al*., 1991). The shorter chain fatty acids ($C_{4:0}$ to $C_{12:0}$) are present in lower quantities on a weight basis, they are primarily responsible for the piquant flavour of hard Italian cheeses, such as Parmesan and Romano, or the sharp goaty/sheep-like flavours of soft goat milk cheeses (Guinee and O'Brien, 2010). The structure of triacylglycerides is illustrated in Fig. 1.

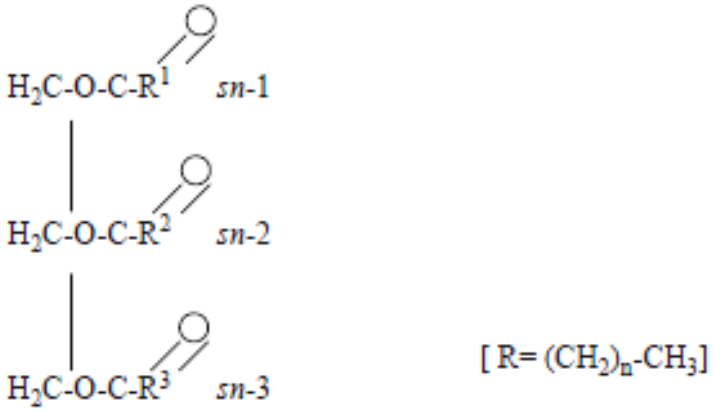

Fig. 1 Triacylglyceride structure (Fox *et al.*, 2000).

Triacylglycerides are esters of glycerol composed of a glycerol backbone with three fatty acids attached (Stryer, 1988). Positioning of fatty acids on the triacylglyceride is non-random; the sn-position of a fatty acid denotes its position on the triacylglyceride. Fatty acids may be esterified at positions 1, 2 or 3 as shown in Fig. 1. $C_{4:0}$, and $C_{6:0}$ are predominately located at the sn-3 position and the sn-1 and sn-3 positions, respectively. As chain length increases up to $C_{16:0}$, an increasing proportion is esterified at the sn-2 position. $C_{18:0}$ is generally located at the sn-1 position, while unsaturated fatty acids are esterified mainly at the sn-1 and sn-3 positions (Balcao and Malcata, 1998).

2. AGENTS OF LIPOLYSIS IN CHEESE

Many authors have reported that cheeses made from skim milk, or milk in which milk fat had been replaced by other lipids did not develop correct flavour (Foda *et al*., 1974; El-Safty and Isamil, 1982; Wijesundera *et al*., 1998). Therefore, milk fat is an essential source of flavour in cheese during ripening. Lipids, in fact, are source of fatty acids, especially short-chain fatty acids, which have strong and characteristic flavours. In this regard, fatty acids are released by the action of lipases in a process referred to as lypolisis (Fox *et al*., 2000). In some varieties, the fatty acids may be converted to other sapid and aromatic compounds, especially methyl ketones and lactones. The enzymatic hydrolysis of triacylglycerides to fatty acids and glycerol, mono- or diacylglycerides (lipolysis) is essential to flavour development in some cheese varieties (McSweeney and Sousa, 2000). As mentioned before, lipolysis in cheese is due to the presence of lipolytic enzymes, which are hydrolases that cleave the ester linkage between a fatty acid and the glycerol core of the triacylglyceride, releasing FFA, and mono- and diacylglycerides (Deeth and Touch, 2000). Lipolytic enzymes may be classified as esterases or lipases, which are distinguished according to three main characteristics: (1) length of the hydrolysed acyl ester chain, (2) physico-chemical nature of the substrate and (3) enzymatic kinetics. Esterases hydrolyse acyl ester chains between 2 and 8 carbon atoms in length, while lipases hydrolyse those acyl ester chains of 10 or more carbon atoms. Esterases hydrolyse soluble substrates in aqueous solutions while lipases hydrolyse emulsified substrates. The enzymatic kinetics of esterases and lipases also differ; esterases have classical Michaelis–Menten type kinetics while lipases, since they are activated only in the presence of a hydrophobic/hydrophilic interface, display interfacial Michaelis–Menten type kinetics (Chich *et al*., 1997). Unfortunately, the terms ‘‘esterases’’ and ‘‘lipases’’ are often used interchangeably in the scientific literature. Free fatty acids are released upon lipolysis and contribute directly to cheese flavour, especially short- and intermediate- chain FFA (Bills and Day, 1964). In general, lipolytic enzymes are specific for the outer ester bonds of tri- or diacylglycerides (i.e., sn-1 and sn-3 positions) (Deeth and Touch, 2000). Lipases in cheese originate from three possible sources: (1) the milk, (2) rennet preparation (rennet paste), (3) starter and non-starter bacteria and, possibly, (6) their addition as exogenous lipases (Deeth and Fitz-Gerald, 1995; Fox and Wallace, 1997; McSweeney and Sousa, 2000).

2.1 MILK LIPASES

Milk contains a very potent endogenous lipoprotein lipase (LPL), which is well characterized (Olivecrona and Bengtsson-Olivecrona, 1991; Olivecrona *et al*., 1992). The enzyme is present in milk due to leakage through the mammary cell membrane from the blood where it is involved in the metabolism of plasma triacylglycerides. Bovine milk contains 10–20 nM L^{-1} lipase which, under optimum conditions (37°C, pH 7) with addition of an apolipoprotein activator, apo-CII, could theoretically release sufficient FFA acids within 10 s to cause perceptible hydrolytic rancidity. Hydrolysis of as little as 1-2% (w/ v) of the milk triacylglycerides to fatty acids gives a rancid or ‘‘lipolysed’’ flavour to the milk (Olivecrona and Bengtsson-Olivecrona, 1991). This does not occur under normal circumstances as LPL and fat are compartmentalized; ca. 90% (w/v) LPL in milk is associated with the casein micelles and the fat, occurring in globules, is surrounded by a lipoprotein membrane (MFGM). If the MFGM is damaged, e.g., due to agitation, foaming, homogenization, inappropriate milking or milk-handling techniques, significant lipolysis may occur resulting in off-flavours in cheese and other dairy products (Darling and Butcher, 1978; Deeth and FitzGerald, 1978; Fox *et al*., 2000). LPL displays a preference for hydrolysis of medium-chain triacylglycerides (MCT) with a 2 fold increase in the rate of hydrolysis of MCT emulsions containing $C_{6:0}$, $C_{8:0}$, $C_{10:0}$ or $C_{12:0}$ esterified FA compared to long chain triacylglyceride (LCT) emulsions containing esterified $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, or $C_{20:0}$ (Deckelbaum *et al*., 1990). LPL has been shown to be relatively non-specific for fatty acid type, but is specific for the sn-1 and sn-3 positions of mono, di- and triacylglycerides (Olivecrona *et al*., 1992). Therefore, short- and medium-chain fatty acids are preferentially released by LPL. LPL is of more significance in raw milk cheeses than in cheeses made from pasteurized milk, since its activity is not reduced by pasteurization. According to Deeth and FitzGerald (1983), it is generally accepted that high-temperature short-time (HTST) treatment (72°C for 15 s) inactivates the enzyme very extensively. However, it is still thought to contribute to lipolysis in pasteurized-milk cheese, as 78°C x 10 s is required for its complete inactivation (Driessen, 1989).

2.2 LIPASES IN RENNET

Commercial rennets are normally free from lipolytic activity. However rennet paste, used in the manufacture of some hard Italian varieties (e.g., Provolone, Romano), contains the lipase, pregastric esterase (PGE), which is responsible for the extensive lypolysis in and the characteristic “piccante” flavour of such varieties (Nelson *et al*., 1977). PGE, also called

lingual or oral lipase, is secreted by glands at the base of the tongue. Suckling stimulates the secretion of PGE, which is subsequently washed into the abomasa by milk and saliva (Fox *et al*., 2000). PGE is highly specific for short chain acids esterified at the sn-3 position (Nelson *et al*., 1977; Fox and Stepaniak, 1993). Rennet paste is prepared from the abomasa of calves, kids or lambs slaughtered after suckling. The specificity of calf, kid and lamb PGEs differ slightly, and consequently the flavour characteristics of cheese differ slightly, depending on the source of PGE (Fox *et al*., 2000).

2.3 MICROBIAL LIPASES

Lactic acid bacteria (LAB), especially *Lactococcus* and *Lactobacillus* spp., are generally considered to be weakly lipolytic in comparison to species such as *Pseudomonas, Acinetobacter* and *Flavobacterium* (Stadhouders and Veringa, 1973; Fox *et al*., 1993; Chich *et al*., 1997). However, in the absence of strongly lipolytic agents and when present at high numbers over a long period, as in ripening cheese, lipases and esterases of lactococci and lactobacilli are probably the principle lypolitic agents in Cheddar and Dutch-type cheeses made from pasteurized milk. The lipases and esterase activity of LAB appears to be exclusively intracellular. Cell-free extracts of various dairy LAB are most active on tributyrin at pH 6-8 and at 37°C. They have little or no activity on triglycerides of long-chain fatty acids (e.g., > C_{10}). Extensive lipolysis occurs in mold-ripened cheese, particularly Blue varieties. Lipolysis in mold-ripened varieties is due primarily to the lipases of *Penicillium roqueforti* or *Penicillium camemberti*, which secrete potent, well-characterized extracellular lipases (Gripon, 1993). *Penicillium camemberti* appears to excrete only one lipase, which is optimally active at around 9.0 and 35°C. *Penicillium roqueforti* excretes two lipases, one with a pH optimum at around 8.0, the other at around 6.0. The acid and alkaline lipases exhibit different specificities (Charton *et al*., 1992; Sidebottom *et al*., 1991).

3. CATABOLISM OF FATTY ACIDS

In cheese, FFA released as a result of lipolysis, especially short- and medium- chain fatty acids directly contribute to cheese flavour. FFA also act as precursor molecules for a series of catabolic reactions leading to the production of flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols (Gripon *et al*.,1991; Fox and Wallace, 1997; McSweeney and Sousa, 2000). Pathways of fatty acid catabolism are outlined in Fig. 2.

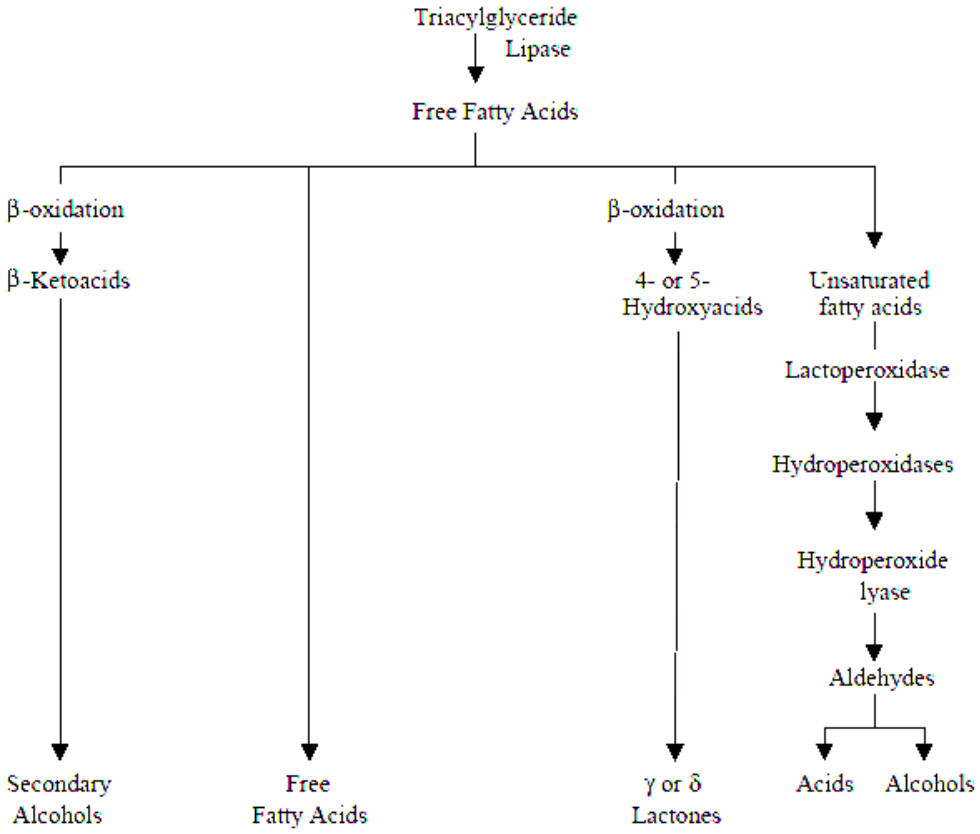

Fig. 2 Catabolism of free fatty acids (FFA) (Molimard and Spinnler, 1996).

Methyl ketones (alkan-2-ones) are important fatty acid catabolites which are formed in cheese due to the action of mould lipases, e.g., *Penicillium roqueforti* (Urbach, 1997), *Penicillium camemberti* and *Geotrichum candidum* (Lawrence, 1966; Lamberet *et al.*, 1982; Cerning *et al.*, 1987; Molimard and Spinnler, 1996). Spores, as well as vegetative mycelia, have been shown to produce methyl ketones (Chalier and Crouzet, 1998). Methyl ketones are the most important flavour components present in Blue cheese and they are present at the highest concentrations. The pathway by which methyl ketones are formed is referred to as β-oxidation. The steps include: release of FFA by lipases, oxidation of the released FFA to α-ketoacids, decarboxylation of keto acids to alkan-2-ones, of less carbon atom, followed by the reduction of alkan-2-ones to the corresponding alkan-2-ol. Furthermore, it has been suggested that fatty acids are not the only methyl ketone precursors (Dartey *et al.*, 1973; Kinsella and Hwang, 1976a). It has been shown that methyl ketones can be formed also by mould cultures from the ketoacids naturally present at low concentrations in milk fat or by oxidation of monounsaturated fatty acids (Kinsella and Hwang, 1976b). The rate of production of methyl ketones in cheese is affected by temperature, pH, physiological state of the mould and the concentration of fatty acids.

Esters and thioesters are other products of fatty acid catabolism, and are common components of cheese volatiles (Urbach, 1997). A great diversity of esters is present in cheese (Molimard and Spinnler, 1996). Esters are highly flavoured and are formed when FFA react with alcohols. Esterification reactions resulting in the production of esters occur between short- to medium chain fatty acids and the alcohols derived from lactose fermentation or from amino acid catabolism. Ethyl esters arise from esterification of ethanol with acetylcoenzyme A (Yoshioka and Hashimoto, 1983). For example, *Geotrichum candidum* is able to produce esters, some of which have a very pronounced melon odour (Jollivet *et al*., 1994). *Pseudomonas fragi* hydrolyses milk fat and esterifies certain of the lower fatty acids with ethanol, producing fruity flavours. Similar esters have been identified in some lactic cultures used in the manufacture of Cheddar cheese (Molimard and Spinnler, 1996). Ethyl, methyl, propyl and butyl esters of even $C_{2:0}$ to $C_{10:0}$ fatty acids have been reported in various cheese varieties (Meinhart and Schreier, 1986). Instead, thioesters are formed when FFA react with free sulphydryl groups (Molimard and Spinnler, 1996).

Secondary alcohols can be formed in cheeses by enzymatic reduction of methyl ketones (Engels *et al*., 1997). *Penicillium* spp. are responsible for the production of secondary alcohols (e.g., 2-pentanol, 2-heptanol and 2-nonanol) in blue-veined cheese due to reduction of methyl ketones (Martelli, 1989).

Lactones are cyclic compounds (Fox *et al*., 1993) formed by the intramolecular esterification of hydroxy fatty acids (Christie, 1983), through loss of water, and the resultant formation of a ring structure (Molimard and Spinnler, 1996). Basic studies of lactone formation in milk fat have showed that lactones are produced by heat, in the presence of water, from their precursor hydroxyacids (Eriksen, 1976). α- and β-lactones are highly reactive and unstable in cheese (Fox and Wallace, 1997). In contrast, however, γ- and δ-lactones are stable and have been identified in cheese; they have 5- and 6- sided rings, respectively. The precursors of lactones, hydroxyacids, in freshly drawn milk are formed in the mammary gland by oxidation of fatty acids (Eriksen, 1976). It has been reported that the mammary glands of ruminants have an δ-oxidation system for fatty acid catabolism (Fox *et al*., 2000). It has been reported that lactones may be formed from keto acids after reduction to hydroxyacids (Wong *et al*., 1975). γ- and δ-lactones can also be formed spontaneously from the corresponding γ- and δ-hydroxyacids following their release from triacylglycerides by lipolysis (Eriksen, 1976); the concentration of these lactones in cheese should, therefore, correlate with the extent of lipolysis. $C_{12:0}$ lactones may be formed by *P. roqueforti* spores and vegetative mycelium from long-chain saturated fatty acids ($C_{18:1}$ and $C_{18:2}$) (Chalier and Crouzet, 1992). Hydroxylation of fatty

acids can also result from normal catabolism of fatty acids. Lactones may also be generated from unsaturated fatty acids by the action of lipoxygenases or hydrolases (Dufossé *et al*., 1994). The potential for lactone production depends on such factors as feed, season, stage of lactation and breed (Fox *et al*., 2000).

Finally, aldehydes are formed from amino acids by transamination, resulting in the formation of an imide that can be decarboxylated. It is also proposed that aldehydes are originated by Strecker degradation of amino acids (Keeney and Day, 1957). Aldehydes may also be formed microbially. It has been reported that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* possess the enzyme, threonine aldolase, which can catalyse the direct conversion of threonine and glycine to acetaldehyde (Marshall and Cole, 1983; Wilkins *et al*., 1986). However some straight-chain aldehydes, e.g., butanal, heptanal and nonanal, may be formed as a result of the boxidation of unsaturated fatty acids. Straight-chain aldehydes are characterized by ‘‘green grass-like’’ aromas (Moio *et al*., 1993).

4. CONTRIBUTION OF LIPOLYSIS AND METABOLISM OF FFA TO CHEESE FLAVOUR

The flavour of mature cheese is the result of a series biochemical changes that occur in the curd during ripening, caused by the interaction of starter bacteria, enzymes from the milk, enzymes from the rennet, residual lipase activity and secondary microbial flora (Urbach, 1997). Lipid hydrolysis results in the formation of FFA, which may, directly, contribute to cheese flavour and also serve as substrates for further reactions producing highly flavoured catabolic products. As well as being a source of flavour compounds, it has been proposed that the fat in cheese provides a fat-water-protein interface for flavour forming reactions to occur. Fat also acts as a solvent for fat-soluble flavour compounds, allowing their retention in cheese and release during consumption (Manning, 1974; Olson and Johnson, 1990; Lawrence *et al*., 1993; Wijesundera and Drury, 1999). In this respect, the physical presence of fat in cheese is important for flavour development. Long-chain FFA (> 12 carbon atoms) are considered to play a minor role in cheese flavour due to their high perception thresholds (Molimard & Spinnler, 1996).While short and intermediate-chain, even-numbered fatty acids ($C_{4:0}$-$C_{12:0}$) have considerably lower perception thresholds and each gives a characteristic flavour note. Butanoic acid contributes ‘‘rancid’’ and ‘‘cheesy’’ flavours. Hexanoic acid has a ‘‘pungent’’, ‘‘blue cheese’’ flavour note, octanoic acid has a ‘‘wax’’, ‘‘soap’’, ‘‘goat’’, ‘‘musty’’, ‘‘rancid’’ and ‘‘fruity’’ note. Depending on their concentration and perception threshold,

volatile fatty acids can either contribute positively to the aroma of the cheese or to a rancidity defect. The flavour effect of FFA in cheese is regulated by pH. In cheeses with a high pH, e.g., surface bacterially ripened cheese, the flavour effect of fatty acids may be affected by the neutralization (Molimard & Spinnler, 1996). The release of secondary metabolites is of great importance to cheese flavour. Given suitable conditions of maturation, these compounds will enhance the flavour complexity (Nicol and Robinson, 1999). The secondary metabolites resulting from lipolysis include: methyl ketones, lactones, esters and secondary alcohols. Methyl ketones are responsible for the unique flavour of Blue cheese, especially, heptan-2-one and nonan-2-one (Jolly and Kosikowski, 1975). Fatty acids and secondary alcohols are also major flavour components (Arnold *et al*., 1975; King and Clegg, 1979). Octan-2-one, nonan-2-one, decan-2-one, undecan-2-one and tridecan-2-one are described as having ‘‘fruity’’, ‘‘floral’’ and ‘‘musty’’ notes, heptan-2-one has a blue cheese note (Rothe *et al*., 1982). The mushroom and musty notes of methyl ketones are important contributors to the flavour of Camembert cheese (Molimard & Spinnler, 1996). According to Eriksen (1976) lactones possess a strong flavour. Although the aromas of lactones are not cheeselike, they may contribute to overall cheese flavour (Fox *et al*., 1993; Fox and Wallace, 1997; Fox *et al*., 2000) and have been reported to impart a buttery character in cheese (Dirinck and De Winne, 1999). δ-Lactones have low flavour thresholds compared to other volatile flavour compounds (O’Keefe *et al*., 1969) and are generally characterized by very pronounced, fruity notes (‘‘peach’’, ‘‘apricot’’ and ‘‘coconut’’) (Dufossé et al., 1994). δ-Lactones have generally higher detection thresholds than those of γ-lactones. Thresholds are relatively low for γ-octalactone, γ-decalactone and γ-dodecalactone (0.7–1.1 mg 100 g^{-1} in water) and even lower for shorter chain lactones (Dufossé et al., 1994). It has been reported that esters are important contributors to the flavour of Parmigiano-Reggiano cheese (Meinhart and Schreier, 1986). In a survey of various cheese varieties, Engels *et al*. (1997) found high concentrations of ethyl butanoate in cheeses with a ‘‘fruity’’ note such as Gruyere, Parmesan and Proosdij. This fruity flavour, which arises due to esters, is considered undesirable in Cheddar cheese (Urbach, 1997; McSweeney and Sousa, 2000). Thioesters often have characteristic aromas in many foods, e.g., in onions, garlica nd some fruits (Cavaille-Lefebvre *et al*., 1998) and Law (1984) reported that thioesters have a ‘‘cheesy’’ aroma. Arora *et al*. (1995) analysed the odour-active volatiles in Cheddar cheese headspace and found that most of the esters separated had a ‘‘buttery’’ to ‘‘fruity’’ aroma. However, thioesters formed by the reaction of esters of short-chain fatty acids with methional imparted the characteristic ‘‘cheesy’’ aroma to Cheddar cheese. According to Lamberet *et al*. (1997), S-methyl thioesters contribute a

characteristic strong flavour to various smear-ripened soft cheeses (e.g., Tilsit, Limburger and Havarti). Secondary alcohols, also resulting from lipolysis, may contribute to cheese flavour (Arora *et al*., 1995). Propan-2-ol, butan-2-ol, octan-2-ol and nonan-2-ol are encountered in most soft cheeses and are typical components of the flavour of Blue cheeses (Engels *et al*., 1997).

5. MATERIALS AND METHODS

5.1 STANDARD AND REAGENTS

Triton X-100, p-nitrophenyl-acetate (pNa), p-nitrophenyl propinate (pNp), p-nitrophenyl butyrate (pNb), p-nitrophenyl caprate (pNc), p-nitrophenyl laurate (pNl), p-nitrophenyl myristate (pNm), p-nitrophenyl palmitate (pNpa), p-nitrophenyl stearate (pNs), β-naphthyl acetate (βNa), β-naphthyl propionate (βNp), β-naphthyl butyrate (βNb), β-naphthyl laurate (βNl), β-naphthyl myristate (βNm), β-naphthyl palmitate (βNPa), β-naphthyl stearate (βNs), β-naphthyl oleate (βNo), fluorescein dyacetate (FDA), glycine, trizma base, sodium dodecyl sulphate (SDS), glycerol, ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylene bis-acrylamide, and acrylamide (all electrophoretic grade), were supplied by Sigma Aldrich (Milan, Italy). Fast Blue BB salt and fluorescein sodium salt were obtained from Fluka (Buchs, Switzerland); fluorescein dibutyrate (FDB), fluorescein dicaproate (FDC) and fluorescein dilaurate (FDL) were supplied by ICN Biomedicals Inc (Aurora, Ohio). Disodium N-Lauryl Iminodipropionate (Deriphat 160) was purchased by Cognis S.p.A. Care Chemicals (Fino Mornasco, Como).

5.2 PLANT MATERIALS

The extract of *Cynara cardunculus* L. (CC) was purchased by Prodor (Bobbio, Piacenza, IT). To obtain a representative sample, latex was collected from *Ficus carica* L. trees, under the same environmental and soil characteristics, in the Puglia region (Foggia, Italy). All samples were harvested manually at the same stage of maturation (August 2010), by incising the stalk of the green fruit from the main branch. Several drops of the latex from the fig were allowed to drip into the test tubes. It was clarified by centrifugation at 14000g at 4°C for 15 min. The clear supernatant (CS) was separated from the gummy material (GM) and stored at –20°C until further use.

5.3 PROTEIN DETERMINATION

Protein concentration was determined by the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein.

5.4 DETECTION OF LIPASE AND ESTERASE ACTIVITY ON PLATES

Lipase/esterase activity on solid media was detected according to the method of Lomolino *et al.* (2012), with some modifications. Fluorescein acetate (FDA), fluorescein dibutyrate (FDB), fluorescein dicaproate (FDC) and fluorescein dilaurate (FDL) were used for the visualization of enzymatic activity. Plates were filled with 2% (w/v) agarose dissolved in tris buffer saline (TBS) at pH 7.2. The solution was cooled to 70°C and 20 mg of each fluorescent substrate, dissolved in 1 mL of acetone, was added. A volume of 25 mL of this buffer solution was transferred into the plates and cooled at room temperature. Wells of 3 mm of diameters were obtained using a cork borer with a volume per well of approximately 20 µL. To obtain calibration curve, increasing amount of fluorescein (0.3, 0.45, 0.6 and 0.75 µg), dissolved in TBS, were loaded in each well. Similarly, plant samples were loaded into each well and heat-inactivated enzyme was used as control. Plates were closed by lids, sealed with parafilm, and incubated at 40°C for 5 h in dark conditions. Circular areas, surrounding each well, were visible under UV light. The images of enzymatic activity were acquired by a UV transluminator (Gel Logic 112 Imaging System). Fluorescent diameters were taken using Carestream Molecular Imaging Software (Mac & PC) v5.0.2. Data collected were converted in arbitrary units (A.U.): µg fluorescein formed/5 h/µg of protein.

5.5 LIPASE AND ESTERASE ACTIVITY ASSAY

Lipase and esterase activity was determined according to the method of Lomolino *et al.* (2003), with some modifications. Activity was measured using p-nitrophenyl and β-naphthyl esters with different chain lengths as substrates. 10% (w/v) and 20% (w/v) of Triton X-100 were used to dissolve the required amount of p-nitrophenyl and β-naphthyl esters, respectively. The reaction mixture containing 1.350 mL tris buffer saline (TBS) pH 7.2, 0.05 mL of synthetic ester and 0.1 mL of enzymatic extract was incubated at 37°C for 60 min. Blank was prepared by adding heat-inactivated enzyme to the standard mixture. After enzymatic reaction, 0.2 mL of a solution 2 mg/mL of Fast BB salt was added, as dye coupler, to the mixture containing β-naphthyl esters at 25°C for 15 min. The released p-nitrophenol and β-naphthol were spectrophotometrically monitored at 400 and 500 nm, respectively, using a Varian Cary 50 Bio UV/VIS spectrophotometer. Standard curves were obtained with a

known concentration of p-nitrophenol and β-naphthol dissolved, respectively, in 10% (w/v) and 20% (w/v) of Triton X-100 and treated as for the samples. Results were expressed in arbitrary units (A.U.): μg p-nitrophenol or β-naphthol formed/60min/μg of protein under the assay conditions.

5.5.1 Effect of pH and Temperature on Lipase and Esterase Activity

The effect of pH on lipase and esterase activity was determined at 37°C within the range of pH 3.0-10.0. The buffers used were 100 mM acid sodium citrate (pH 3.0-6.0), tris buffer saline (pH 7.2), 100 mM Tris-HCl (pH 8.0-9.0), 100 mM Na-carbonate (pH 10.0). Similarly, an analysis of the effect of temperature on the lipase and esterase activity of the plant enzymes was carried out to determine the temperature optimum. Enzyme samples were incubated at different temperatures in the range of 20-80°C at their optimal pH. Activity measurements were performed as described above.

5.6 Electrophoretic Analyses

All electrophoretic procedures were carried out at room temperature in a Mini-Protean II apparatus (Bio-Rad Laboratories, Milano, Italy).

SDS-PAGE was performed according to Laemmli (1970) with a total polyacrylamide concentration of 12% (w/v). Samples to be electrophoresed were treated with denaturing buffer 1.33 M Tris (pH 7.4), glycerol 40% (v/v) and SDS 8% (w/v). SDS-PAGE was carried out at 100 V until the tracking dye bromophenol blue ran off the gel. Gels were used to detect esterase activity.

Native-PAGE (N-PAGE) in 10% (w/v) total polyacrylamide gels was performed as SDS-PAGE, but the detergent SDS was omitted from both the gel and the running buffer. Samples were added of glycerol, bringing the glycerol to a final concentration of 10% (w/v), and loaded on the gel. N-PAGE was carried out at 80 V. Gels were used for esterase activity detection.

Deriphat-PAGE (D-PAGE) was performed (Rizzi *et al.*, 2003) as N-PAGE, but in the presence of 0.1% (w/v) disodium N-Lauryl Iminodipropionate (Deriphat 160) in the cathodic buffer (12.5 mM Tris, 96 mM glycine). D-PAGE was carried out at 80 V. Gels were used for zymographic analysis.

For two dimensional-PAGE (2D-PAGE), a lane of gel, in which the serum of *Ficus carica* (CS) had been separated by D-PAGE, was cut out and heated at 100°C for 5 min in a buffer made of 8% (w/v) of SDS, 5 mL glycerol and 1 mL of 1.33 M Tris pH 7.4, containing 8%

(w/v) SDS. After cooling at room temperature, the gel was inserted horizontally on the top of a 12% (w/v) SDS-PAGE gel and electrophoresed as described for SDS-PAGE.

5.7 Zymogram Analysis

After electrophoresis, gels were washed exhaustively two times for 15 min in TBS, pH 7.2. Lipase/esterase activity was detected by incubation in 5 mL of 20% (w/v) Triton X-100 containing 25 mg of β-naphthyl acetate, propionate, butyrate and Fast Blue BB salt at 40°C for 45 min. The activity was observed as deep red bands over a yellow background.

5.8 Statistical Analysis

ORAC data are presented as means ± SD. Statistical analysis was carried out using statistical package Statgraphics Centurion XVI (StatPoint Technologies Inc., USA, 2010) program and significance of each group was verified with the analysis of the One-way ANOVA followed by the Tuckey test at $p < 0.05$.

6. Results and Discussion

In the present study esterase/lipase activity of *Cynara cardunculus* L. and *Ficus carica* L. was characterized. Several methods have been developed for the measurement of lipases in crude or purified lipase preparations (Hasan *et al*., 2009). For example, agar plate assays, which are useful for studying and quantifying enzymes present in biological extracts (Zocca *et al.*, 2008), are an interesting alternative to other methods. At first, esterase/lipase activity of these two plant extracts was studied by using an agar plate method (Lomolino *et al.*, 2012) in presence of fluorescein acetate (FDA), dibutyrate (FDB), dicaproate (FDC) and dilaurate (FDL). As described previously, *Ficus carica* latex was clarified by centrifugation to separate the clear supernatant (CS) from the gummy material (GM). Both two phases were employed only in this preliminary test to detect similarities and differences in their enzymatic activity. Fig. 3, 4, 5 show esterase activity of *Cynara cardunculus* (CC) and *Ficus carica* in its liquid (CS) and gummy material (GM) on FDA, FDB, FDC and FDL. The enzymatic activity in the presence of these fluorescent substrates revealed that plant extracts possessed esterase and not lipase activity. Esterases would be defined as enzymes having optimum activity on esters containing C_2 to C_4 fatty acids while lipases are those enzymes with optimum activity on esters containing fatty acids with six or more carbons (Kilcawley *et al*., 1998). In particular, the catalytic activity was observed for short chain substrates (acetate and butyrate) (Fig. 3) but

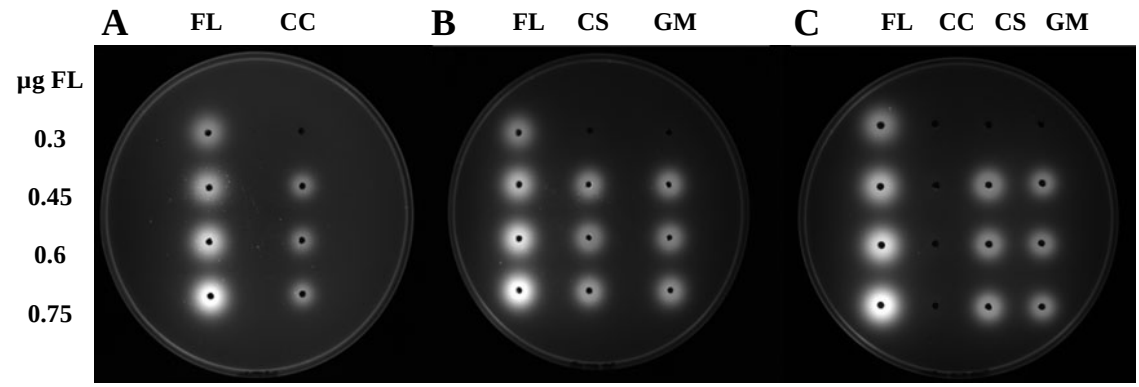

Fig. 3 Esterase activity on plates containing fluorescein diacetate (FDA, plates A and B) and fluorescein dibutyrate (FDB, plate C) in presence of *Cynara cardunculus* (CC), clear supernatant (CS) and gummy material (GM) of *Ficus carica*. The calibration curve was obtained with increasing doses of fluorescein (FL) in the same plate.

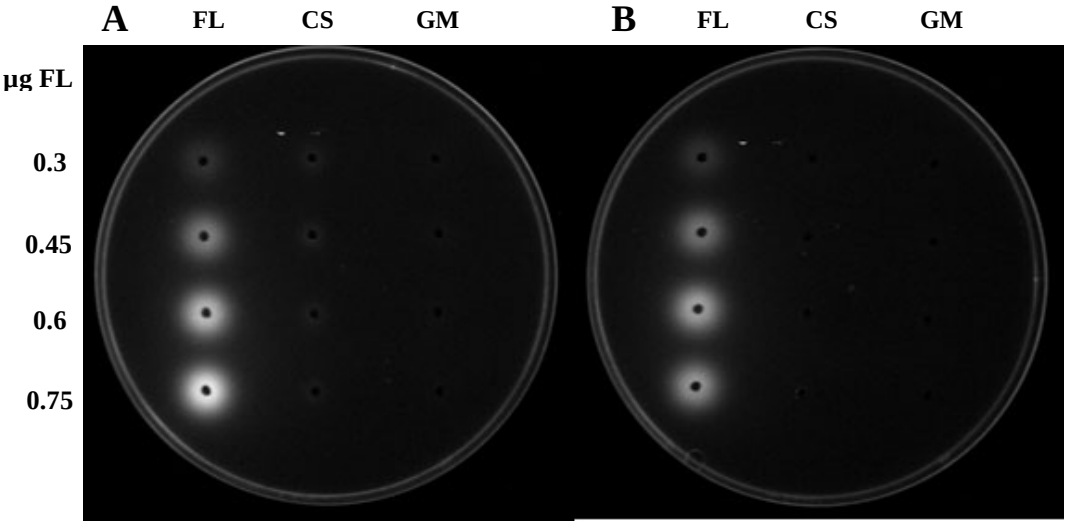

Fig. 4 Esterase activity on plates containing fluorescein diacetate (FDC, plates A) and fluorescein dilaurate (FDL, plate B) in presence of clear supernatant (CS) and gummy material (GM) of *Ficus carica*. The calibration curve was obtained with increasing doses of fluorescein (FL) in the same plate.

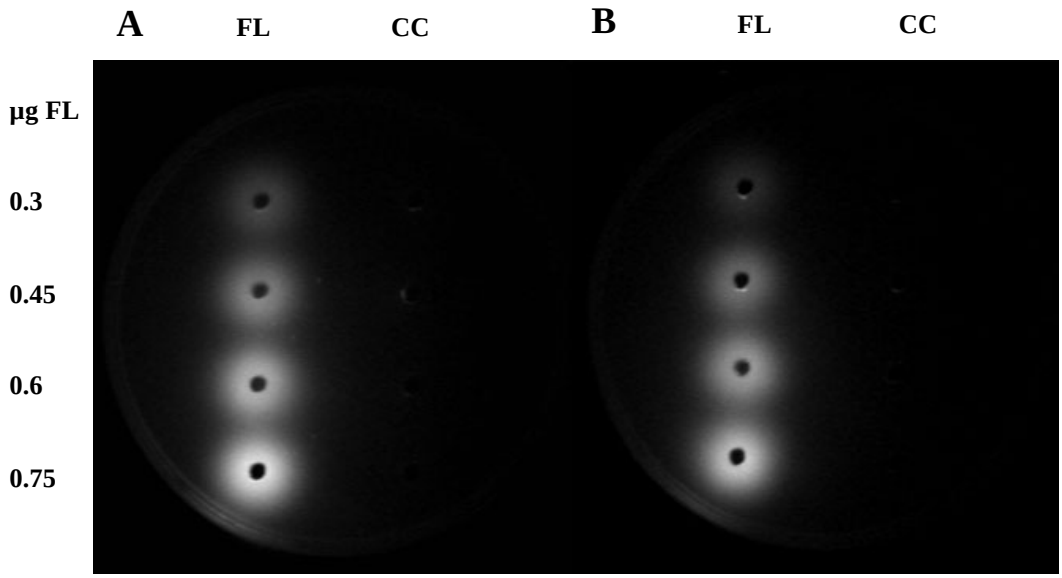

Fig. 5 Esterase activity on plates containing fluorescein diacetate (FDC, plates A) and fluorescein dilaurate (FDL, plate B) in presence of *Cynara cardunculus* (CC). The calibration curve was obtained with increasing doses of fluorescein (FL) in the same plate.

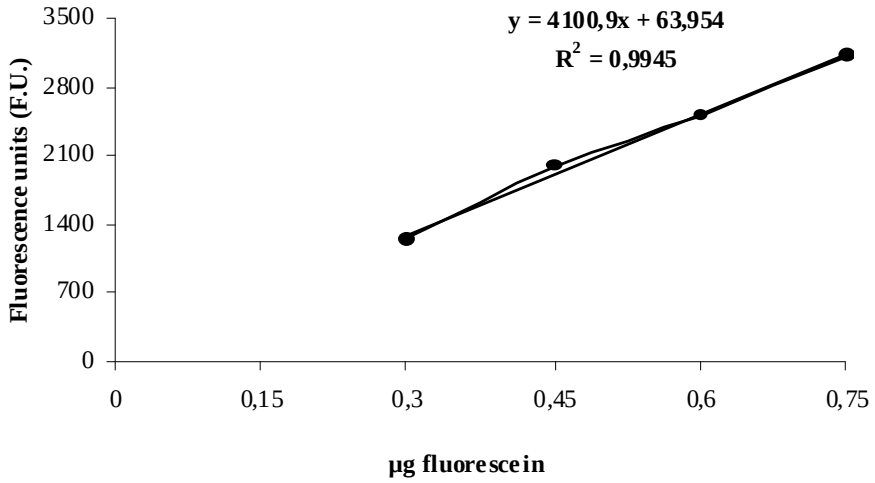

Fig. 6 Standard curve between the fluorescence units (F.U.), expressed by the measure of the height of the peaks, and the increasing doses of fluorescein (0.3, 0.45, 0.6, 0.75 µg).

Table 2 Specific activity of *Cynara cardunculus* (CC), clear supernatant (CS) and gummy material (GM) of *Ficus carica* in presence of fluorescein diacetate (FDA) and fluorescein dibutyrate (FDB). *The activity was expressed as arbitrary units (A.U.): µg fluorescein formed/5 h/ µg of protein. The results are as the means ± SD (n = 3).

Esterase	Fluorescein of:			
	Acetate	Butyrate	Caproate	Laurate
CC	0,0097 ± 0,00035*	-	-	-
CS	0,00526 ± 0,00035*	0,00572 ± 0,00029*	-	-
GM	0,00463 ± 0,00023*	0,00491 ± 0,00010*	-	-

not for long chain ones (caproate and laurate) (Fig. 4-5). The quantification of esterase activity was possible by using the intensity of fluorescence converted into *counts* (Fluorescence Units, F.U.). Therefore, the realization of a calibration curve between F.U. and increasing doses of fluorescein in the same plate (Fig. 6), allowed to calculate the specific activity of these esterases, expressed as arbitrary units (A.U.): µg fluorescein formed/5 h/µg of protein (Table 1). Overall, the esterolytic activity of *Ficus carica* (CS and GM) was significantly higher than *Cynara cardunculus,* which only hydrolyzed FDA. Although CS and GM are two distinct phases of *Ficus carica* latex, separable by centrifugation, they reported approximately the same enzymatic activity in this assay. For example, the esterolytic activity of CS and GM in presence of FDA was 0.00526 and 0.00463 A.U., respectively. These data were slightly lower than those one obtained in the hydrolysis of FDB. However, these results seem to support the concept that, as CS and GM esterases acted similarly, they can be considered the same enzyme with the same biochemical characteristics. Therefore, only CS was chosen for the next enzymatic characterizations.

Subsequently, esterolytic/lypolitic activity of *Cynara cardunculus* and *Ficus carica* was tested *in vitro* by screening two different chromogenic compounds as substrates. The aim of this characterization was to examine the substrate specificity of these enzymes at different pHs and temperatures. A commonly used procedure for investigating esterase and lipase activity makes use of synthetic substrates, such as p-nytrophenyl and β-naphthyl esters with aliphatic acyl chains of various lengths. The hydrolysis of these carboxylic esters leads to the release of alcohols that can be monitored continuously and quantitatively using a spectrophotometric method (Stuer *et al.*, 1986; Vorderwülbecke *et al.*, 1992). The cleaveage of p-nitrophenyl esters releases p-nitrophenol, a yellow product of reaction, while the red one

of β-naphthol derived from β-naphthyl esters can be monitored after complexing the solution with a diazonium salt (Fast Blue BB salt). The enzymatic activity in presence of these substrates again revealed that CC and CS possessed esterase and not lipase activity (Tables 3 and 4). The temperature optimum of esterases/lipases is regarded as one of the most important characteristics for industrial purposes. Therefore, the enzymatic characterization was studied, at first, in terms of temperature optimum. The effect of temperature on CC esterase on p-nitrophenyl and β-naphtyl esters, in the range of 20-80°C, are shown in Fig. 7 and 8. It was found that the enzyme hydrolyzed ester bonds of acetate rather than propionate, with greater affinity for β-naphthyl esters than p-nitrophenyl esters. For example, esterase activity on β-Na and p-Na was 22.6 and 5.37 A.U., respectively, at 50°C. Therefore, the hydrolysis of β-Na, at this temperature, was 17.2 % higher than p-Na. However, no activity was detected on the other fatty acids with four (butyrate) or more carbons, confirming the previous agar plate assay. Overall, CC showed optimum temperature at 50°C for both synthetic esters, but also at 40°C on p-nitrophenyl esters. Above and below these temperatures, CC esterase was not particularly active, which suggested that this enzyme was inhibited at high and low temperatures.

Table 3 Esterase activity of *Cynara cardunculus* (CC) and the clear supernatant (CS) of *Ficus carica* on p-nitrophenyl esters.

Esterase	**p-Nitrophenyl-esters of:**							
	Acetate	Propionate	Butyrate	Caprate	Laurate	Myristate	Palmitate	Stearate
CC	+	+	-	-	-	-	-	-
CS	+	+	+	-	-	-	-	-

Table 4 Esterase activity of *Cynara cardunculus* (CC) and the clear supernatant (CS) of *Ficus carica* on β-naphtyl esters.

Esterase	**β-Naphtyl-esters of:**							
	Acetate	Propionate	Butyrate	Laurate	Myristate	Palmitate	Stearate	Oleate
CC	+	+	-	-	-	-	-	-
CS	+	+	+	-	-	-	-	-

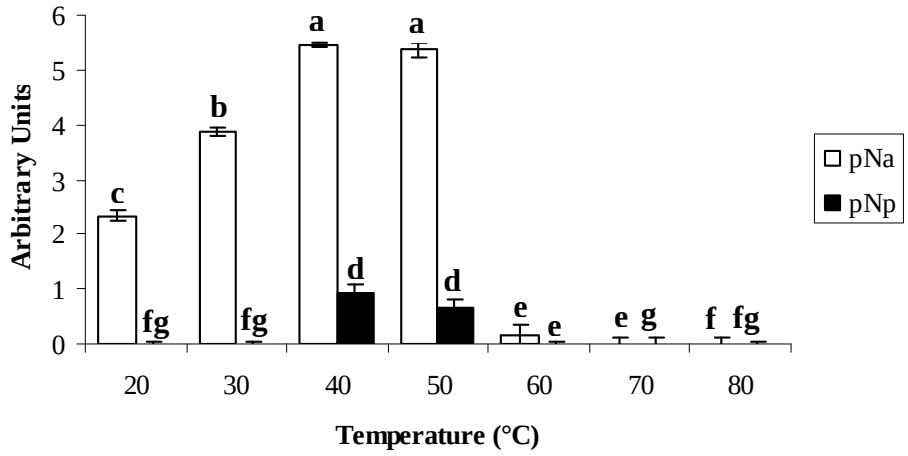

Fig. 7 Effect of temperature on *Cynara cardunculus* (CC) esterase activity. Esterolytic activity was measured using p-nitrophenyl acetate (pNa) and propionate (pNP) as substrates. The results are the means ± SD (n=3).

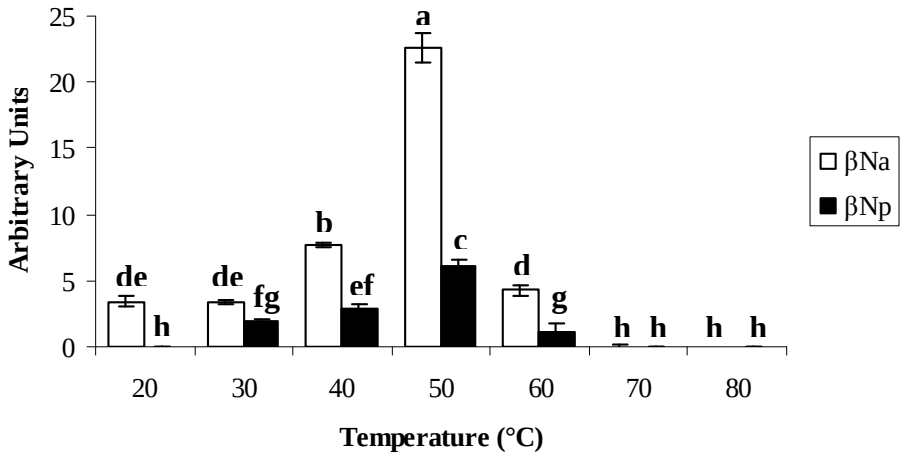

Fig. 8 Effect of temperature on *Cynara cardunculus* (CC) esterase activity. Esterolytic activity was measured using β-naphtyl acetate (β-Na) and propionate (βNP) as substrates. The results are the means ± SD (n=3).

Although in literature no studies report on esterase of *Cynara cardunculus* L. or any other species belonging to *Compositae* family (*Asteraceae*), these results could be comparable to those reported for other plants species. The temperature profile exhibited by CC esterase is similar to that determined in the case of *Jatropha curcas* L. (Staubmann *et al.*, 1999) and other plant esterases (James and Smith, 1974; Daood and Al-Ani, 1988; Rajeshwaran and Prakash, 1995). On the basis of these findings, the CC esterase can be considered as an enzyme with medium temperature optimum.

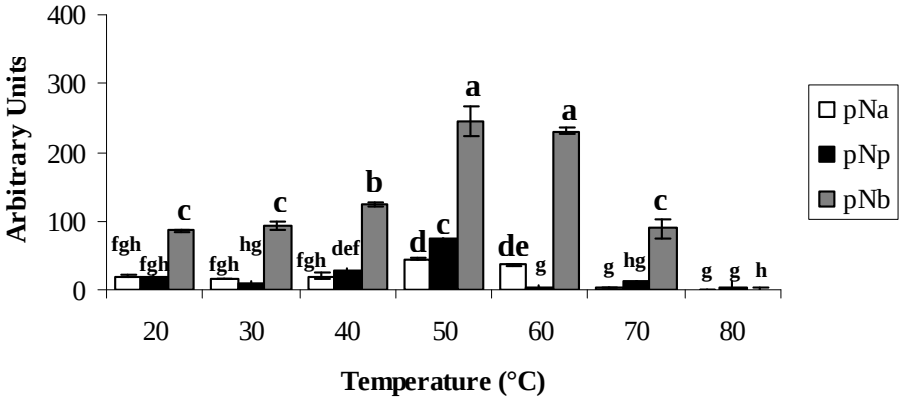

Fig. 9 Effect of temperature on *Ficus carica* L. (CS) esterase activity. Esterolytic activity was measured using p-nitrophenyl acetate (pNa), propionate (pNp) and butyrate (pNb) as substrates. The results are the means ± SD (n=3).

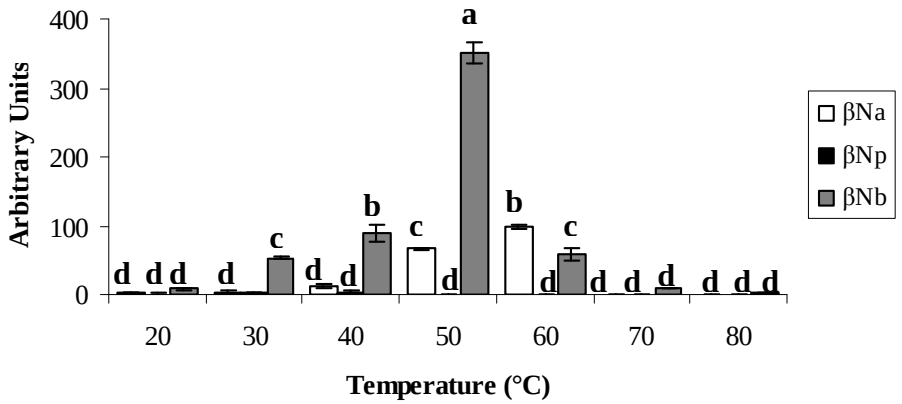

Fig. 10 Effect of temperature on *Ficus carica* L. (CS) esterase activity. Esterolytic activity was measured using β-naphtyl acetate (βNa), propionate (βNp) and butyrate (βNb) as substrates. The results are the means ± SD (n=3).

The results obtained in the evaluation of the temperature optimum of *Ficus carica* (CS) are reported in Fig. 9 and 10. *Ficus carica* showed a greater specificity for p-nitrophenyl esters than β-naphtyl esters. In particular, CS reported the highest activity with butyric acid esters. The maximum value was obtained from the hydrolysis of βNb (351.5 A.U.) at 50°C, followed by pNb at 50°C and 60°C with values of 245.7 and 230.7 A.U., respectively. Besides, CS had the temperature optimum in the range from 50 to 60°C and, high and low temperatures

inhibited the esterolytic activity. Scientific information about fig latex esterases is very limited. To our knowledge a previous study carried out by Visco (1924), reports on the esterases of *Ficus carica* L. The author tested these enzymes by titration of trybutyrin and monobutyrin. Nevertheless, our data could be compared with those of other laticiferous plant species. In this regard, Giordani *et al.* (1991) and Villeneuve (1997) have demonstrated the presence of lipases in the latex of some species of *Asclepiadaceae*, *Euphorbiaceae*, and *Caricaceae*. Although the physiological role of these lipases in the latex has not yet been demonstrated, it was proposed that lipases are involved in both the synthesis and in the hydrolysis of terpenic esters during the formation of latex particles (Giordani *et al.*, 1991). Caro *et al.* (2000) found maximal activity at 45°C for *Euphorbia characias* latex lipase, using tributyrin as substrate. Other authors (Dhiuque-Mayer *et al.*, 2001; Abdelkafi *et al.*, 2011) carried out lipolysis reactions by involving *Carica papaya* latex lipase, which had optimum temperature at 50°C. In general, thermal profiles exhibited by CC and CS were nearly similar to those of esterases used for flavor formation of cheeses from different species of *Pseudomonas* spp. with a temperature optimum ranged from 30 to 70°C (Stead, 1986), as well as *Lactococcus lactis* subsp. *lactis* with maximum activity at 55°C (Chich *et al*., 1997) Therefore, esterases present in these plant coagulants could become very important for improving flavor characteristics of dairy products.

Together with temperature, pH is another important parameter capable of altering and modulating enzymatic activity in aqueous solution. In this regard, biochemical properties of CC and CS were also studied within the range of pH 3.0-10.0, in order to identify the best buffered conditions of these enzymes. Fig. 11 reports the effect of pH on CC esterase on pNa, pNp, βNa and βNp. It was generally observed that these esterases act at neutral pH values. CC esterase showed maximum activity at pH 7.5 on pNa and pNp, while in range between 7.5 and 8.0 on βNa and βNp. Furthermore, the esterolytic activity is higher on acetate esters rather than propionate, confirming the results obtained with the thermal profiles. The optimum pH for CS esterase on p-nitrophenyl and β-naphtyl esters are showed in Fig 12 and 13. Optimum conditions for *Ficus carica* esterase were set at pH 7.5 for propionate and butyrate esters, while at pH 8.0 for acetate esters. The hydrolysis for p-nitrophenyl and β-naphtyl esters proceeded in this way: pNb > pNp > pNa and βNb > βNp and βNa, respectively. Besides, these results were similar to those showed by the esterases of *J. curcas* L. (Staubmann *et al.*, 1999) and rice bran (Prabhu *et al.*, 1999). Finally, the optimum pHs for different plant lipases have been generally reported in the basic pH values (7.0–9.0), and these ranges change depending on enzyme source and substrate (Isbilir, 2008).

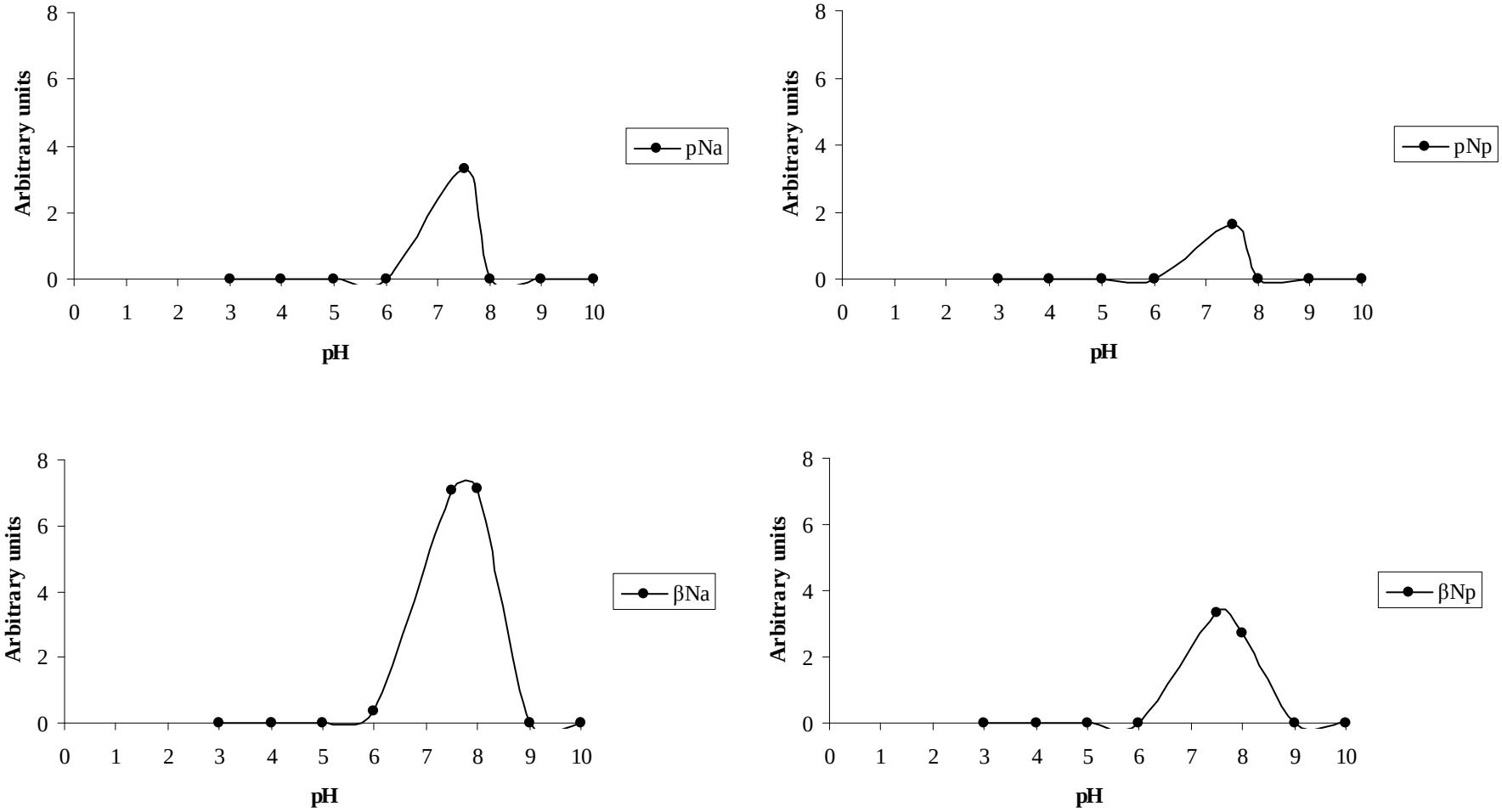

Fig. 11 Effect of pH on *Cynara cardunculus* L. (CC) esterase activity. Esterolytic activity was measured using p-nytrophenyl acetate (pNa), p-nytrophenyl propionate (pNp), β-naphtyl acetate (β-Na) and β-naphtyl propionate (β-Np) as substrates.

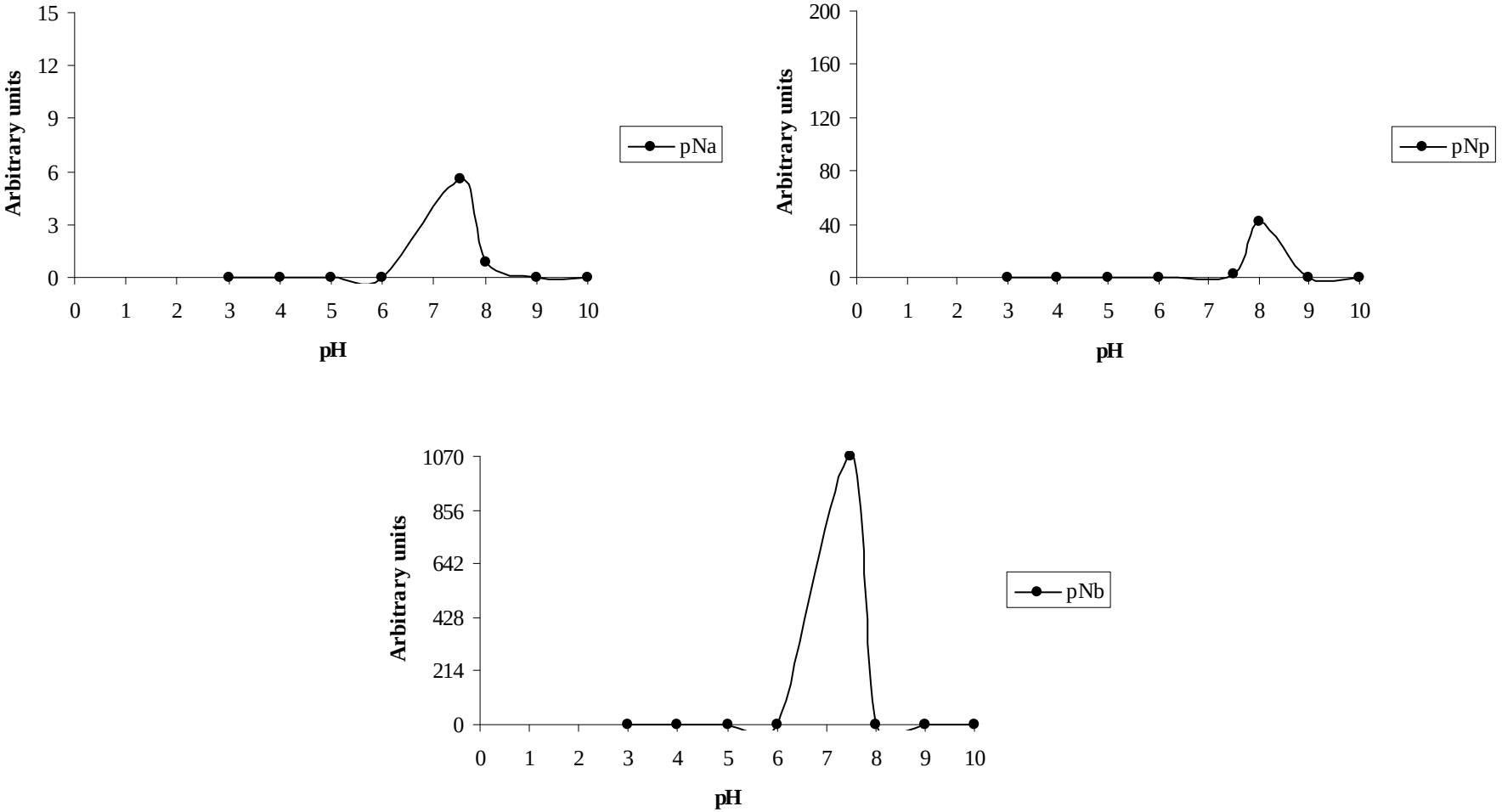

Fig. 12 Effect of pH on *Ficus carica* L. (CS) esterase activity. Esterolytic activity was measured using p-nytrophenyl acetate (pNa), propionate (pNp) and butyrate (pNb) as substrates.

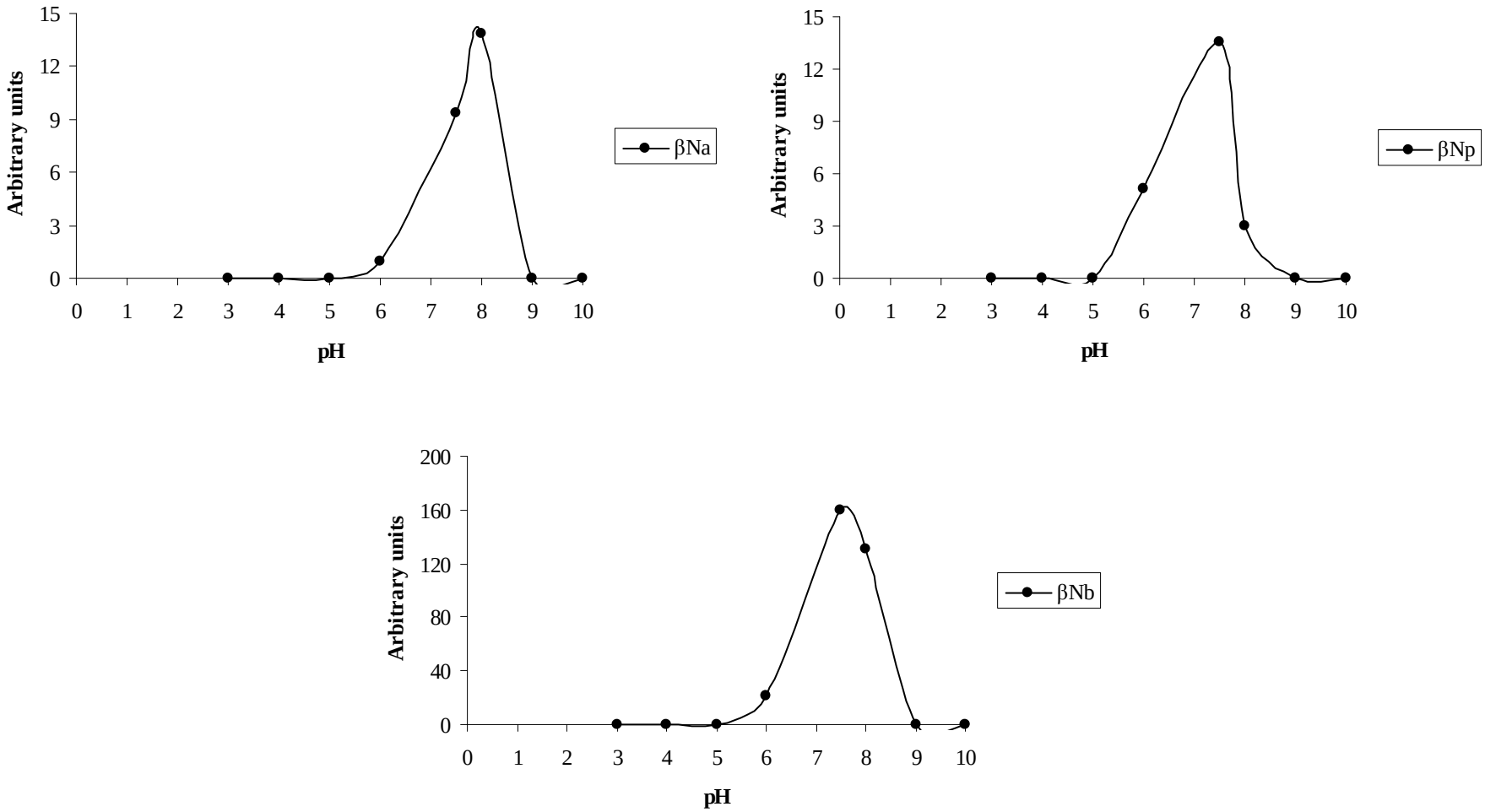

Fig. 13 Effect of pH on *Ficus carica* L. (CS) esterase activity. Esterolytic activity was measured using β-naphtyl acetate (β-Na), propionate (β-Np) and butyrate **(**β-Nb) as substrates.

Moreover, some species of *Pseudomonas* spp., used for flavor formation of cheeses, has showed a pH optimum ranged from 7 to 9 (Stead, 1986). A similar range of pH (7-8) was also reported for *Lactococcus lactis* subs. *lactis* esterase (Chich *et al*., 1997). In this regard, the esterolytic activity of these plant samples could be considered during cheese ripening when pH begins rising.

A common analytical technique for protein identification is its electrophoretic separation and subsequent detection on polyacrylamide gels (Singh *et al*., 2006). In this regard, zymography is one of the widely used techniques to study the functionality of enzymes. Thus, protein(s) responsible for the esterolytic activity were identified, at first, by SDS-PAGE, then by native PAGE. SDS-PAGE, which normally allows the proteins separation in function of their molecular weight, can be successfully used for the activity staining of lipase after removing SDS from the gel (Sommer *et al.*, 1997). However, it works best for monomeric enzymes. Some SDS-sensitive proteins and enzymes with multisubunits can be denatured irreversibly. This may lead to the loss of activity of the enzyme (Saminathan, 2008).

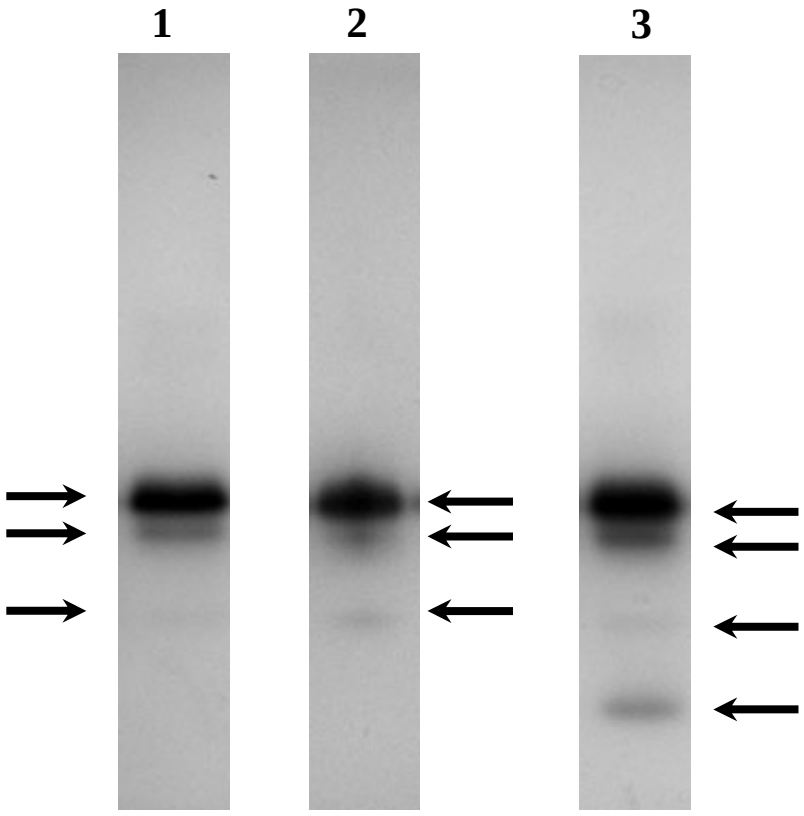

Fig. 14 Esterase activity of *Ficus carica* L. (CS) after SDS-PAGE and detected with different substrates: β-naphthyl acetate (lane 1), propionate (lane 2) and butyrate (lane 3).

In this regard, several attempts were made to detect the esterolytic activity of CC on SDS-PAGE but without results. This seems to support the concept that CC is denaturated by SDS or it is formed by multisubunits. On the other hand, zymography of CS esterase showed that the activity of the resolved enzyme was preserved even in presence of SDS. Fig. 14 reports the zymogram obtained performing SDS-PAGE of CS esterase in presence of βNa, βNp and βNb. The arrows indicate the bands of proteins corresponding to the esterase activity. The eletrophoretic pattern displayed by this esterase is different in relation to the substrate used. For example, the use of β-naphtyl acetate and propionate (lane 1 and 2) generated activity profiles differing from those obtained with the β-naphtyl butyrate. In particular, CS showed three different bands with a similar intensity and eletrophoretic migration in presence of βNa and βNp, while it displayed four bands with βNb. This result suggests the presence of enzymes acting specifically on esters with different chain lenghts, therefore it appears to confirm the hypothesis that CS esterase has more isoforms. Furthermore, the zymogram analysis validates the previous assay with β-naphtyl esters and, in particular, the finding that CS had more affinity and activity on βNb. The zymographic technique was also performed in native conditions. Traditional native electrophoresis is limited in its applicability to native protein analysis because of high or low operative pH which may adversely affect proteins. Another drawback in native gel electrophoresis is the need for separate acidic and basic gels for resolution of enzymes. Traditional native gel electrophoresis is limited by its incompatibility with native samples that aggregate (Shuen-Fuh, 1996). In the present study, the native eletrophoresis of esterase proteins of *Cynara cardunculus* were not detected, perhaps for the small dimension of the enzyme or for its particular chemical nature that prevents it from maintaining its activity during electrophoresis. Whereas, *Ficus carica* generated aggregates which produced vertical streaking. Other authors (Rizzi *et al*., 2003) reported the same trouble with the membrane-bound esterase of *Saccharomyces cerevisiae*, whose enzymatic proteins appeared at the top of the gel. This could be due to the absence of a detergent within the electrophoretic system, causing the insolubilization of the enzyme molecule, when it had just entered the gel. Thus, it seems that these esterases presented insoluble parts or idrophobic aminoacids and/or lipids which, in the aqueous electrophoretic conditions, were difficultly separable. Some authors have developed methods to solve aggregation of lipases seen in native PAGE, i.e., Saminathan *et al*. (2008) which modified blue native polyacrylamide gel electrophoresis (PAGE) protocol. However, in the present study it was necessary the use of a surfactant in order to find the conditions allowing the enzyme to remain soluble during the electrophoretic migration. In this regard, Markwell *et al.*

(1979), Gregory *et al.* (1982), Rizzi *et al.* (2003) obtained satisfactory results by including Deriphat in the electrophoretic system. Deriphat 160 (disodium N-Lauryl Iminodipropionate) is a zwitterionic detergent which dissolves hydrophobic proteins without denaturation. Therefore, Deriphat was included in the gel and cathodic buffer as well as it is normally done for sodium dodecyl sulfate during SDS-PAGE. This method allowed to detect CS esterase activity. In a first attempt, CS sample was electrophoresed in presence of Deriphat, but the strong activity of the enzyme, did not allow a good visualization and resolution of the esterase isoforms. For this reason it was necessary to dilute the sample. The zymogram of CS esterase (Fig. 15) on βNa displayed a marked band and another one less intense; thus, the enzymatic activity was the expression of two isoforms.

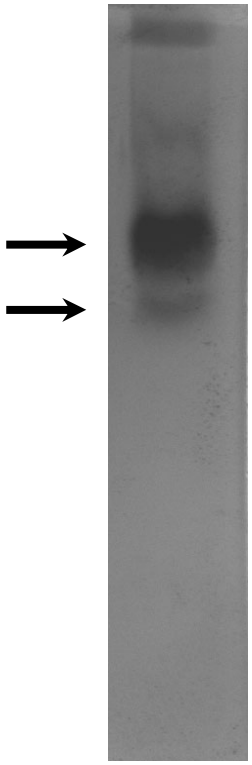

Fig. 15 Esterase activity of *Ficus carica* L. (CS) after Deriphat PAGE (D-PAGE) and detected with β-naphthyl acetate.

Finally, SDS-PAGE and D-PAGE were also used as the first electrophoretic system in bidimensional PAGE (2D-PAGE), allowing the resolution of single bands into several spots and the detection of isoforms. As shown in Fig. 16, the main and the minor bands detectable in native and denaturing conditions were not divided into additional isoforms.

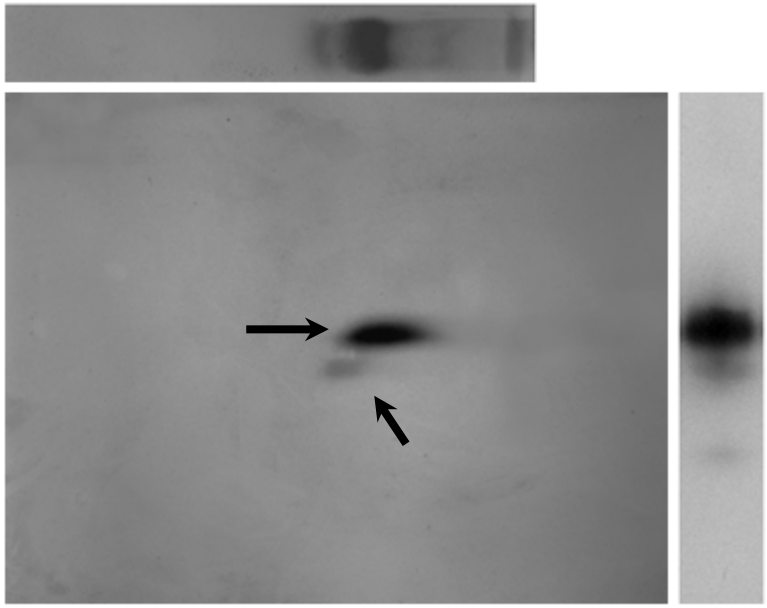

Fig. 16 Esterase activity of *Ficus carica* L. (CS) after bidimensional PAGE (2D-PAGE) and detected with β-naphthyl acetate. First dimension (top side): Deriphat PAGE. Second dimension (right side): SDS-PAGE.

Therefore, it can be assumed that the main isoform represents a protein with a strong enzymatic activity. This study could be a preliminary work and starting point for further isolation and sequencing of the enzyme.

7. CONCLUSIONS

In summary, this chapter reports on the characterization of esterase/lipase activity of *Cynara cardunculus* L. and *Ficus carica* L. The biochemical properties of these esterases, i.e., substrate specificity, pH and temperature, are useful for exploring the potentials applications of these plant esterases in dairy industry. In this regard, further studies need to be carried out to assess the use of *Cynara cardunculus* and *Ficus carica* esterases in cheese making and their contribution on lypolysis and development of flavour.

8. REFERENCES

Abdelkafi, S., Barouh, N., Fouquet, B., Fendri, I., Pina, M., Scheirlinckx, F., Villeneuve, P. and Carrière, F. (2011) '*Carica papaya* Lipase: A Naturally Immobilized Enzyme with Interesting Biochemical Properties', *Plant Foods for Human Nutrition*, 66, 34-40.

Arnold, R. G., Shahani, K. M. and Dwivedi, B. K. (1975) 'Application of lipolytic enzymes to flavour development in dairy products', *Journal of Dairy Science*, 58, 1127-1143.

Arora, G., Cormier, F. and Lee, B. (1995) 'Analysis of odor-active volatiles in Cheddar cheese headspace by multidimensional GC/MS/sniffing', *Journal of Agricultural and Food Chemistry*, 43, 748-752.

Balcao, V. M. and Malcata, F. X. (1998) 'Lipase catalysed modification of milk fat', *Biotechnology Advances*, 16, 309-341.

Banks, W. (1991) 'Milk lipids', in *Utilizations of milk fat*, Brussels: International Dairy Federation, Bulletin 260, 3-6.

Bills, D. D. and Day, E. A. (1964) 'Determination of the major free fatty acids of Cheddar cheese.', *Journal of Dairy Science*, 47, 733-738.

Bornscheuer, U.T. (2002*)* 'Microbial Carboxyl esterases: classification, properties and application in biocatalysis', *FEMS Microbiology Reviews*, 26, 73–81.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemestry*, 72, 248-254.

Caro, Y., Villeneuve, P., Pina, M., Reynes, M. and Graille, J. (2000) 'Lipase activity and fatty acid typoselectivities and plant extracts in hydrolysis and interesterification', *Journal of the American Oil Chemists' Society*, 77, 349-354.

Cattaneo, T. M. P., Nigro, F., Messina, G. and Giangiacomo, R. (1994) 'Effect of an enzymatic complex from pineapple pulp on the primary clotting phase', *Milchwissenschaft*, 49, 269-272.

Cavaille-Lefebvre, D., Combes, D., Rehbock, B. and Berger, R. G. (1998) 'A chromatographic and mass-spectrometric approach for the analysis of lipase produced thioester derivatives', *Applied Microbiology and Biotechnology*, 49, 136-140.

Cerning, J., Gripon, J. C., Lamberet, G. and Lenoir, J. (1987) 'Les activités biochimiques des *Penicillium* utilisés en fromagerie', *Le Lait*, 67, 3-39.

Chalier, P. and Crouzet, J. (1992) 'Production of lactones by *Penicillium roqueforti*', *Biotechnology Letters*, 14, 275-280.

Chalier, P. and Crouzet, J. (1998) 'Methyl ketone production from copra oil by *Penicillium roqueforti* spores', *Food Chemistry*, 63, 447-451.

Charton, E., Davies, C. and McCrae, A. R., (1992) 'Use of specific polyclonal antibodies to detect heterogeneous lipases from *Geotrichum candidum*', *Biochimica Biophysica Acta*, 1127, 191-198.

Chazarra, S., Sidrach, L., Lopez-Molina, D. and Rodriguez-Lopez, J. (2007) 'Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus*, L.) flowers', *International Dairy Journal*, 17, 1393-1400.

Chich, J. F., Marchesseau, K. and Gripon, J. C. (1997) 'Intracellular esterase from *Lactococcus lactis* subsp. *lactis* NCDO 763: Purification and characterization', *International Dairy Journal*, 7, 169-174.

Christie, W. W. (1983) 'The composition and structure of milk lipids', in P. F. Fox, ed., *Developments in dairy chemistry-2*, London: Applied Science Publishers, 1-35.

Daood, H. and Al-Ani, H. (1988) 'Biochemical properties of safflower lipase' *Acta Alimentaria*, 37, 127-139.

Darling, D. F. and Butcher, D. W. (1978) 'Milk-fat globule membrane in homogenized cream', *Journal of Dairy Research*, 45, 197-208.

Dartey, C., Kinsella, K. and Kinsella, J. E. (1973) 'Metabolism of [U-14C] lauricac id to methyl ketones by the spores of Penicillium roqueforti', Journal of Agricultural and Food Chemistry, 21, 933-936.

Deckelbaum, R. J., Hamilton, J. A., Moser, A., Bengtsson-Olivecrona, G., Butbul, E., Carpentier, Y. A., Gutman, A. and Olivecrona, T. (1990) 'Medium-chain versus long chain triacylglycerol emulsion hydrolysis by lipoprotein lipase and hepaticlipase: Implications for the mechanisms of lipase action', *Biochemistry*, 29, 1136-1142.

Deeth, H. C. and Fitz-Gerald, C. H. (1978) 'Effects of mechanical agitation of raw milk on the milk-fat globule in relation to the level of induced lipolysis', *Journal of Dairy Research*, 45, 373-380.

Deeth, H. C. and Fitz-Gerald, C. H. (1983) 'Lipolyticenzymes and hydrolytic rancidity in milk and milk products', in Fox, P. F., ed., *Developments in dairy chemistry-2*, Essex: Applied Science Publishers, 195-240.

Deeth, H. C. and Fitz-Gerald, C. H. (1995) 'Lipolyticenzymes and hydrolytic rancidity in milk and milk products', in Fox, P. F., ed., *Advanced dairy chemistry-2-Lipids*, London: Chapman & Hall, 247-308.

Deeth, H. C. and Touch, V. (2000) 'Methods for detecting lipase activity in milk and milk products', *Australian Journal of Dairy Technology*, 55, 153-168.

Dhuique-Mayer, C., Caro., Y., Pina., M., Ruales, J., Dornier., M. and Graille, J. (2001) 'Biocatalytic properties of lipase in crude latex from babaco fruit (*Carica pentagona*)' *Biotechnology Letters*, 23,1021-1024.

Dirinck, P. and De Winne, A. (1999) 'Flavour characterisation of cheeses by gas chromatographic-mass spectrometric profiling', *Journal of Chromatography A*, 847, 203-208.

Driessen, F. M. (1989) 'Heat-induced changes in milk. Bulletin 238', Brussels: International Dairy Federation.

Duarte, A. R., Duarte, M. D. R., Moreira, K. A., Cavalcanti, M. T. H., de Lima, J. L. and Porto, A. L. F. (2009) '*Jacaration corumbensis* O. Kuntze a new vegetable source for milk-clotting enzymes' *Brazilian Archives of Biology and Technology*, 52, 1-9.

Dufossé, L., Latrasse, A. and Spinnler, H. E. (1994) 'Importance des lactones dans les arômes alimentaires: Structures, distribution, propriétés sensorielles et biosynthèse', *Science Alimentaire*, 14, 17-50.

Egito, A. S., Girardet, J. M., Laguna, L. E., Poirson, C., Mollé, D., Miclo, L., Humbert, G. and Gaillard, J. L. (2007) 'Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k-casein', *International Dairy Journal*, 17, 816-825.

El-Safty, M. S. and Isamil, A. A. (1982) 'Addition of free fatty acids to improve the flavour and accelerate the ripening of Domiati cheese made from reconstituted milk', *Journal of Dairy Research*, 49, 671-675.

Engels, W. J. M., Dekker, R., de Jong, C., Neeter, R. and Visser, S. (1997) 'A comparative study of volatile compounds in the watersoluble fraction of various types of ripened cheese', *International Dairy Journal*, 7, 255-263.

Eriksen, S. (1976) 'Flavours of milk and milk products. I. The role of lactones', *Milchwissenschaft*, 31, 549-550.

Fadyloglu, S. (2001) 'Immobilization and characterization of ficin', *Nahrung / Food*, 45, 143-146.

Foda, E. A., Hammond, E. G., Reinbold, G. W. and Hotchkiss, D. K. (1974) 'Role of fat in flavour of Cheddar cheese', *Journal of Dairy Science*, 57, 1137-1142.

Fox, P. F., Law, J., McSweeney, P. L. H. and Wallace, J. (1993) 'Biochemistry of cheese ripening', in Fox, P. F., ed., *Cheese: Chemistry, physics and microbiology (2nd ed.), Vol. 1*, London: Chapman & Hall, 389-438.

Fox, P. F. and Stepaniak, L. (1993) 'Enzymes in cheese technology' *International Dairy Journal*, 3, 509-530.

Fox, P. F and Wallace, J. M. (1997) 'Formation of flavour compounds in cheese', *Advances in Applied Microbiology*, 45, 17-85.

Fox, P. F. and McSweeney, P. L. H., (1998) *Dairy chemistry and biochemistry* London: Blackie Academic & Professional, an imprint of Thomson Science.

Fox, P. F, Guinee, T. P, Cogan, T. M and McSweeney, P. L. H. (2000) *Fundamentals of Cheese Science*, Gaithersburg Maryland: Aspen Publishers, Inc.

Giordani, R., Moulin A. and Verger, R. (1991) 'Tributyroylglycerol hydrolase activity in *Carica papaya* and other lattices', *Phytochemistry*, 30, 1069-1072.

Gregory, R. P. F., Borbély, G., Demeter, S. and Faludi-Dániel, Á. (1982) 'Chiroptical properties of chlorophyll-protein complexes separated on Deriphat/polyacrylamide gel', *Biochemical Journal*, 202, 25-29.

Gripon, J. C., Monnet, V., Lamberet, G. and Desmazeaud, M. J. (1991) 'Microbial enzymes in cheese ripening', in Fox, P. F., ed., *Food enzymology*, New York: Elsevier Science Publishing Co., Inc., 131-168.

Gripon, J. C. (1993) 'Mould-ripened cheeses', in Fox, P. F., ed., *Cheese: Chemistry, physics and microbiology (2nd ed.), Vol. 2*, London: Chapman & Hall, 111-136.

Guinee, T. P. and O'Brien, B. (2010) 'The Quality of Milk for Cheese Manufacture', in Law, B. A. and Tamime, A. Y., eds., *Technology of cheesemaking*, 2nd ed., New Delhi: Wiley Blackwell, 98-129.

Gunstone, F. D., Harwood, J. L. and Padley, F. B. (1994) 'Milk lipids', in *The lipid handbook*, 2nd ed., London: Chapman & Hall, 195-197.

Hasan, F., Shah, A. A. and Hameed, A. (2009) 'Methods for detenction and characterization
of lipase: A comprehensive review', *Biotechnology Advances*, 27, 782-798.

Huppertz, T., Kelly, A. L. and Fox, P. F. (2009) 'Milk lipids-composition, origin and properties', in Tamime, A. Y., ed., *Dairy Fats and Related Products*, Oxford: Wiley-Blackwell, 1-27.

Isbilir, S. S., Ozcan, H. M. and Yagar, H. (2008) 'Some biochemical properties of lipase from Bay Laurel *(Laurus Nobilis* L.*)* Seeds', *Journal of the American Oil Chemists' Society*, 85, 227-233.

James, D. J. and Smith, A. R. W. (1974) 'A biochemical study of non-specific esterases from plant cells, employing the histochemical substrate naphtol AS-D acetate', *Histochemical Journal*, 6, 7-23.

Jeager, K. E, Dijkstra, B. W. and Reetz, M. T. (1999) 'Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications', *Annual Review of Microbiology*, 53, 315-351.

Jensen, R. G., Gander, G. W. and Sampugna, J. (1962) 'Fatty acid composition of the lipids from pooled, raw milk', *Journal of Dairy Science*, 45, 329-331.

Jensen, R. G., Ferris, A. M. and Lammi-Keefe, C. J. (1991) 'The composition of milk fat', *Journal of Dairy Science*, 74, 3228-3243.

Jensen, R. G. (2002) 'The composition of bovine milk lipids: January 1995 to December 2000', *Journal of Dairy Science*, 85, 295-350.

Jollivet, N., Chateaud, J., Vayssier, Y., Bensoussan, M. and Belin, J. M. (1994) 'Production of volatile compounds in model milk and cheese media by eight strains of *Geotrichum candidum* Link', *Journal of Dairy Research*, 61, 241-248.

Jolly, R. C. and Kosikowski, F. V. (1975) 'Flavour development in pasteurized milk blue cheese by animal and microbial lipase preparations', *Journal of Dairy Science*, 58, 846-921.

Kazlauskas, R. J and Bornscheuer, U. T. (1998) 'Biotransformations with lipases', in Rehm, H. J., Pihler, G., Stadler, A. and Kelly, P. J. W., eds, *Biotechnology*, 8, New York: VCH, 37-192.

Keenan, T. W. and Maher, I. H. (2006) 'Intracellular origin of milk fat globules and the nature of the milk fat globule membrane', in Fox, P.F. and McSweeney, P.L.H., eds., *Advanced Dairy Chemistry, Volume 2: Lipids,* 3rd ed., New York: Springer Science & Business Media, 137-171.

Keeney, M. and Day, E. (1957) 'Probable role of Streker degradation of amino acids in development of cheese flavour', *Journal of Dairy Science*, 40, 874-876.

Kilcawley, K. N., Wilkinson, M. G. and Fox, P. F. (1998) 'Enzyme-modified cheese [a review]', *International Dairy Journal*, 8, 1-10.

King, R. D. and Clegg, G. H. (1979) 'The metabolism of fatty acids, methyl ketones and secondary alcohols by *Penicillium roqueforti* in blue cheese slurries', *Journal of the Science of Food and Agriculture*, 30, 197-202.

Kinsella, J. E. and Hwang, D. H. (1976a) 'Enzymes of *Penicillium roqueforti* involved in the biosynthesis of cheese flavour', *Critical Reviews in Food Science and Nutrition*, 8, 191-228.

Kinsella, J. E. and Hwang, D. H. (1976b) 'Biosynthesis of flavours by *Penicillium roqueforti*', *Biotechnical Biogenesis*, 18, 927-938.

Laemmli, U. K. (1970) 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', *Nature*, 227, 680-685.

Lamberet, G., Auberger, B., Canteri, C. and Lenoir, J. (1982) 'L'Aptitude de *Penicillium caseicolum* à la dégradation oxidative des acides gras', *Revue Laitiere Française*, 406, 13-19.

Lamberet, G., Auberger, B., and Bergere, J. L. (1997) 'Aptitude of cheese bacteria for volatile S-methyl thioester synthesis. II. Comparison of coryneform bacteria, Micrococcaceae and some lactic acid bacteria starters', *Applied Microbiology and Biotechnology*, 48, 393-397.

Law, B. A. (1984) 'Flavour development in cheese', in Davies, F. L. and Law, B. A., eds., *Advances in the microbiology and biochemistry of cheese and fermented milk*, London: Elsevier Applied Science Publishers, 187-208.

Lawrence, R. C. (1966) 'The oxidation of fatty acids by spores of *Penicillium roqueforti*', *Journal of General Microbiology*, 44, 393-405.

Lawrence, R. C., Giles, J. and Creamer, L. K. (1993) 'Cheddar cheese and related dry-salted cheese varieties', in Fox, P. F., ed., *Cheese: Chemistry, physics and microbiology (2nd ed.), Vol. 2*, London: Chapman & Hall, 1-38.

Llorente, B. E., Brutti, C. B. and Caffini, N. O. (2004) 'Purification and characterization of a milk-clotting aspartic proteinase from globe artichoke (*Cynara scolymus*, L.)', *Journal of Agricultural and Food Chemistry*, 52, 8182-8198.

Lomolino, G., Di Pierro, G. and Lante A., (2012) 'A Quantitative Fluorescence-Based Lipase Assay', *Food Technology and Biotechnology*, 50(4), 479-482.

Lomolino, G., Rizzi, C., Spettoli, P., Curioni, A., Lante, A. (2003) 'Cell vitality and esterase activity of Saccharomyces cerevisiae is affected by increasing calcium concentration', *Agro Food Industry Hi-Tech*, 32-35.

Manning, D. J. (1974) 'SuCShur compounds in relation to Cheddar cheese flavour', *Journal of Dairy Research*, 41, 81-87.

Markwell, J. P., Thornber, J. P. and Boggs, R. T. (1979). 'Higher chloroplasts: evidence that all the chlorophyll exists as chlorophyll-protein complexes', *Proceedings of the National Academy of Sciences*, 76 (3), 1233-1235.

Marshall, V. M. and Cole, W. M. (1983) 'Threonine aldolase and alcohol dehydrogenase activities in *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* and their contribution to flavour production in fermented milks', *Journal of Dairy Research*, 50, 375-379.

Martelli, A. (1989) 'Volatiles in the flavour of Gorgonzola cheese', *Rivista Societe Italiana Scienze Alimentari*, 18, 251-262.

McPherson, A. V. and Kitchen, B. J. (1983) ‘Reviews of the progress of dairy science: The bovine milk-fat globule membrane-its formation, composition, structure and behaviour in milk and dairy products’, *Journal of Dairy Research*, 50, 107-133.

McSweeney, P. L. H. and Sousa, M. J. (2000) ‘Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review’, *Le Lait*, 80, 293-324.

Meinhart, E. and Schreier, P. (1986) ‘Study of flavour compounds from Parmigiano Reggiano cheese’, *Milchwissenschaft*, 41, 689-691.

Moio, L., Dekimpe, J., Etievant, P. X. and Addeo, F. (1993) ‘Volatile flavour compounds of water buffalo Mozzarella cheese’, *Italian Journal of Food Science*, 5, 57-68.

Molimard, P. and Spinnler, H. E. (1996). ‘Review: Compounds involved in the flavour of surface mould-ripened cheeses: Origins and properties’, *Journal of Dairy Science*, 79, 169-184.

Nelson, J. H., Jensen, R. G. and Pitas, R. E. (1977) ‘Pregastric esterase and other oral lipases-a review’, *Journal of Dairy Science*, 60, 327-362.

Nicol, K. and Robinson, R. (1999) ‘The taste test’, *Dairy Industries International*, 64, 35-38.

O’Keefe, P. W., Libbey, L. M. and Lindsay, R. C. (1969) ‘Lactones in Cheddar cheese’, *Journal of Dairy Science*, 52, 888.

Olivecrona, T. and Bengtsson-Olivecrona, G. (1991) ‘Indigenous enzymes in milk. II. Lipase’, in Fox, P. F., ed., *Food enzymology, Vol. 1*, London: Elsevier Applied Science, 62-78.

Olivecrona, T., Vilaro, S. and Bengtsson-Olivecrona, G. (1992) ‘Indigenous enzymes in milk. II. Lipases in milk’, in Fox, P. F., ed., *Advanced dairy chemistry. 1. Proteins*, London: Elsevier Applied Science, 292-310.

Olson, N. and Johnson, M. E. (1990) ‘Light cheese products: Characteristics and economics’, *Food Technology*, 44, 93-96.

Prabhu, A. V, Tambe, S. P., Gandhi, N. N, Sawant, S. B. and Joshi, J. B. (1999) ‘Rice bran lipase: extraction, activity and stability’, *Biotechnology Progress* , 15(6), 1083-1089.

Rajeshwara, A. N. and Prakash, V. (1995) ‘Purification and characterization of lipase from rice (*Oryza sativa* L.) bran’, *Die Nahrung*, 39, 406-418.

Raposo, S. and Domingos, A. (2008) ‘Purification and characterization of milk-clotting aspartic proteinases from Centaura calciptrea cell suspension cultures’, *Process Biochemistry*, 43, 139-144.

Rizzi, C., Lomolino, G., Lante, A., Crapisi, A., Spettoli, P. and Curioni, A. (2003) 'Solubilization and activity detection in polyacrylamide gels of a membrane-bound esterase from an oenological strain of *Saccharomyces* cerevisiae', *Journal of the Institute Brewing & Distilling*, 109 (3), 187-193.

Rothe, M., Engst, W. and Erhardt, V. (1982) 'Studies on characterization of blue cheese flavour', *Nahrung*, 26, 591-602.

Salhi-Hannachi, A., Chatti, K., Saddoud, O., Mars, M., Rhouma, A.,Marrakchi, M. and Trifi, M. (2006) 'Genetic diversity of different Tunisian fig (*Ficus carica* L.) collections revealed by RAPD fingeprrints', *Hereditas*, 143, 15-22.

Saminathan, M., Thirumalai Muthukumaresan, K., Srinivas Rengarajan, Nandhini Muthukrishnan and Pennathur Gautam (2008) 'Blue native electrophoresis study on lipase', *Analytical Biochemistry*, 377, 270-271.

Lin, S. F., Chiou, C. M., Yeh, C. M. and Tsai, Y. C. (1996) 'Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111', *Applied and Environmental Microbiology*, 62, 1093-1095.

Sidebottom, C. M., Charton, E., Dunn, P. P. J., Mycick, G., Davies, C., Sutton, J. L., MacCrae, A. R. and Slabas, A. R. (1991) '*Geotrichum candidum* produces several lipases with markedly different substrates specificities', *European Journal of Biochemistry*, 202, 485-491.

Silva, S. V., Barros, R. M. and Malcata, F. X. (2002) 'Hydrolysis of caseins by extracts of *Cynara cardunculus* precipitated by ammonium sulphate', *Journal of Food Science*, 67, 1746-1751.

Singh, R., Gupta, N., Goswami, V. K., Gupta, R. (2006) 'A simple activity staining protocol for lipases and esterases', *Applied Microbiology and Biotechnology*, 70, 679-682.

Sommer, P., Bormann, C. and Gotz, F. (1997) 'Genetic and Biochemical Characterization of a New Extracellular Lipase from *Streptomyces cinnamomeus*', *Applied and Environmental Microbiology*, 63, 3553-3560.

Sousa, J. and Malcata, X. (1997) 'Ripening of ovine milk cheeses: effect of plant rennet, pasteurization, and addition of starter on lypolysis', *Food Chemistry*, 59 (3), 427-432.

Stadhouders, J. and Veringa, H. A. (1973) 'Fat hydrolysis by lactic acid bacteria in cheese', *Netherlands Milk and Dairy Journal*, 27, 77-91.

Staubmann, R., Ncube, I., Gübitz, G. M., Steiner, W. and Read, J. S. (1999) 'Esterase and lipase activity in *Jatropha curcas* L. seeds', *Journal of Biotechnology*, 75, 117-126.

Stead, D. (1986) 'Microbial lipases their characteristics role in food spoilage and industrial uses', *Journal of Dairy Research*, 53, 481-505.

Stryer, L. (1988) 'Biochemistry 3rd ed.', New York: W. H. Freeman and Company.

Stuer, W., Jaeger, K. E. and Winkler, U. K. (1986) 'Purification of extracellular lipase from *Pseudomonas aeruginosa*', *Journal of Bacteriology*, 168, 1070-1074.

Teixeira, G., Santana, A. R., Pais, M. S. and Clemente, A. (2000) 'Enzymes of *Opuntia ficus-indica* (L.) miller with potential industrial applications', *Applied Biochemistry and Biotechnology*, 88, 299-312.

Urbach, G. (1997) 'The flavour of milk and dairy products: II. Cheese: Contribution of volatile compounds', *International Journal of Dairy Technology*, 50, 79-89.

Vairo Cavalli, S., Silva, S. V., Cimino, C., Malcata, F. X. and Priolo, N. (2008) 'Hydrolysis of caprine and ovine milk proteins, brought about by aspartic peptidases from *Silybum marianu* flowers', *Food Chemistry*, 106, 997-1003.

Villeneuve, P., Pina, M., Skarbek, A., Graille, J. and Foglia, T. A. (1997) 'Specificity of *Carica papaya* latex in lipase-catalyzed interesterification reactions', *Biotechnology Techniques*, 11, 91-94.

Visco, S. (1924) 'Sui fermenti esistenti nel lattice del *Ficus carica.* I: Esterasi', *Archivio di Farmacologia Sperimentale e Scienze Affini*, 38, 243-250.

Vorderwülbecke, T., Kieslich, K. and Erdmann, H. (1992) 'Comparison of lipases by different assays', *Enzyme and Microbial Technology*, 14, 631-639.

Wijesundera, C., Drury, L., Muthuku-marappan, K., Gunasekaran, S. and Everett, D. W. (1998) 'Flavour development and the distribution of fat globule size and shape in Cheddar-type cheese made from skim milk homogenized with AMF or its fractions', *Australian Journal of Dairy Technology*, 53, 107.

Wijesundera, C. and Drury, L. (1999) 'Role of milk fat in production of Cheddar cheese flavour using a fat-substituted cheese model', *Australian Journal of Dairy Technology*, 54, 28-35.

Wilkins, D. W., Schmidt, R. H., Shireman, R. B., Smith, K. L. and Jezeski, J. J. (1986) 'Evaluating acetaldehyde synthesis from L-[14C(U)] threonine by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*', *Journal of Dairy Science*, 69, 1219-1224.

Wong, N. P., Ellis, R. and LaCroix, D. E. (1975) 'Quantitative determination of lactones in Cheddar cheese', *Journal of Dairy Science*, 58, 1437-1441.

Yoshioka, K. and Hashimoto, N. (1983) 'Cellular fatty acid and ester formation by brewers' yeast', *Agricultural and Biological Chemistry*, 47, 2287-2294.

Zocca, F., Lomolino, G. and Lante, A. (2008) ‘3,4-Dihydroxyphenylalanine gel diffusion assay for polyphenol oxidase quantification’, *Analytical Biochemistry*, 383, 335-336.

CHAPTER 3

Characterization of proteolytic activity

ABSTRACT

Crude extracts of cardoon flowers (*Cynara cardunculus* L.) and fig latex (*Ficus carica* L.) were investigated as a source of proteolytic enzymes to be used in cheese making as an alternative to calf rennet. *Cynara cardunculus* and *Ficus carica* expressed maximum activity at pH 5.0 and 7.0, respectively, and an optimal temperature at 60°C. The proteolytic activity of the plant enzymes towards bovine caseins was investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and densitogram analysis. The extent of enzyme-mediated proteolysis was much higher for ficin than cardosin and depended on the type and specificity of proteinase, enzyme concentration, type of substrate as well as time of hydrolysis. Finally, the study of fig proteinases by zymography revealed the presence of several isoforms, indicating a high level of polymorphism.

Keywords: proteinase, *Cynara cardunculus* L., *Ficus carica* L., proteolysis, densitometry, zymography

INTRODUCTION

Proteolytic enzymes are widely distributed in animals, plants and microorganisms and catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteinases belong to group 3 (hydrolases), and sub-group 4 (which hydrolyse peptide bonds) (International Union of Biochemistry, 1992). They can be also subdivided into two major groups based on their ability to cleave N- or C- terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases). While aminopeptidases cleave the N-terminal peptide linkage, carboxypeptidases cleave the C-terminal peptide bond (Sumantha *et al*., 2006). Based on the functional group present at the active site, proteinases are further classified into four prominent groups, i.e., serine proteinases, aspartic proteinases, cysteine proteinases, and metalloproteases (Hartley, 1960). Proteinases have a large variety of applications, mainly in the detergent and food

industries. In dairy industry, they are involved in the hydrolysis of Phe_{105}-Met_{106} bond in κ-casein (milk clotting reaction) as well as in the development of flavour by the catabolism of free amino acids (FAA). Proteolysis, in fact, is the most complex and important biochemical event occurring during cheese ripening for several reasons. Proteolysis plays a vital role in the development of: (i) textural changes in the cheese curd, due to breakdown of the protein network, decrease in *aw* through water binding by liberated carboxyl and amino groups and increase in pH (in particular in surface mould-ripened varieties); (ii) direct contribution to flavour and perhaps to off-flavour (e.g., bitterness) of cheese through the formation of peptides and free amino acids (FAA); (iii) liberation of substrates (amino acids) for secondary catabolic changes (e.g., deamination, decarboxylation, transamination, desulphuration, catabolism of aromatic compounds); and (iv) changes to the cheese matrix, which facilitate the release of sapid compounds during mastication (McSweeney and Sousa, 2000). Proteinases originate in cheese from different sources such as type of coagulant, i.e., chimosin, pepsin, microbial or plant proteinases. In this respect, when a potential rennet substitute is studied, it is important to evaluate the degradation patterns of the caseins because of their effect on the yield, consistency, and flavor of the final cheese (Fox, 1989). *Cynara cardunculus* L. and *Ficus carica* L., two crop species widely distributed in Mediterranean countries, are sources of proteolytic enzymes. Although the properties of the crude enzyme preparation may well be derived from a combination of proteins, characterization of these crude extracts was regarded as essential due to their potential use in cheese technology. Therefore, the effects of pH and temperature, the proteolytic activity of these plant extracts were investigated. Moreover, the work aimed to characterize the hydrolysis of of bovine α_s- and β-caseins by SDS-PAGE and densitogram analysis. Finally, in the present chapter it is also given an overview of milk proteins, the main important agents of proteolysis in cheese and their contribution to flavour.

1. MILK PROTEINS

The properties of milk and most dairy products are affected more by the proteins they contain than by any other constituent. The nitrogenous fraction of bovine milk consists of casein, whey protein and non protein nitrogen (urea, proteose-peptones, peptides) at levels of ~78, 18 and 4 g 100 g^{-1}, respectively, of total nitrogen. Casein, which is typically present at a level of 2.5 g 100 g^{-1} in cows' milk, is the main structural protein of both rennet- and acid-induced milk gels. The casein is heterogeneous, comprising four main types: α_{s1}, α_{s2}, β and κ, which represent ~38, 10, 35 and 15 g 100 g^{-1} of the total casein, respectively (Fox and McSweeney, 1998; Fox, 2003; Swaisgood, 2003). Model studies in dilute dispersions indicate that the individual caseins vary in the content and distribution of phosphate; the respective number of (serine) phosphate residues *per*

mole of casein are ~8, 10-13, 5 and 1 for α_{s1}, α_{s2}, β and κ-caseins, respectively. The serine phosphates bind calcium and calcium phosphate, and consequently, different caseins have different calcium-binding properties. Casein in milk exists in the form of spherical-shaped colloid particles (~40-300 nm diameter), known as casein micelles (Fox and Brodkorb, 2008; McMahon and Oommen, 2008). The arrangement of casein within the micelle is such that the interior is mainly occupied by the calcium-sensitive caseins (α_s- and β-) and κ-casein is principally located at the surface, with its hydrophilic C-terminal region (caseinomacropeptide) oriented outwards toward the serum phase in the form of protruding negatively charged hairs, which create an electrokinetic potential of ~-20 mV and confer stability to the micelle by electrostatic repulsion, Brownian movement and a consequent steric repulsion (de Kruif and Holt, 2003; Horne and Banks, 2004). Moreover, the C-terminal region of the κ-casein is glycoslyated to varying degrees (Saito and Itoh, 1992; Mollé and Leonil, 1995; Fox and McSweeney, 1998; Mollé *et al*., 2006), containing galactose, *N*-acetylgalactosamine (GalNAc) and/or *N*-actetylneuraminic (sialic) acid (NANA) (Dziuba and Minkiewicz, 1996). These may further enhance the ability of κ-casein to increase micelle stability by steric impedance and electrostatic repulsion via their contribution to increase in water binding (to carbohydrate moieties) and to negatively charged carboxylic groups (on the NANA molecule). O'Connell and Fox (2000) found that the level of glycosylation of κ-casein and protein surface hydrophobicity increased as a function of micelle size. While a predominant surface location of κ-casein confers stability to the casein micelle in native milk, it renders it susceptible to aggregation/flocculation by processes which reduce the solvency of (and collapse/flatten) the κ-casein hairs or remove them, and thereby enable contact between the more hydrophobic micelle cores. However, the interactions between the micelle cores are modified by many factors, including pH, composition of the serum phase, ionic strength, protein concentration and conditions to which milk is subjected (heat, acidification, ultrafiltration/diafiltration homogenisation, shearing). On the other hand, whey protein in cows' milk is typically~0.6-0.7 g 100 g^{-1}vand consists of four main types - β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulin(s) (Ig) and bovine serum albumin (BSA) at levels of ~54, 21, 14 and 6 g 100 g^{-1} of total. The properties of the individual whey proteins have been extensively reviewed (Mulvihill and Donovan, 1987; Brew, 2003; Fox, 2003; Hurley, 2003; Sawyer, 2003). In milk, they exist as soluble globular proteins and are characterised by a relatively high level of intramolecular disulphide bonding, and β-Lg and BSA each contain one cysteine residue per mole. On heat-induced denaturation, the whey proteins can interact via thiol–disulphide bonds with other whey proteins and with κ-casein. The latter results in the formation of κ-casein/ β-Lg aggregates either at the surface of the casein micelle or in the serum phase or both. The size and location (serum/micelle surface) of these aggregates are affected by

severity of heat treatment of milk, pH at heating, ionic strength, calcium level and casein-to-whey protein ratio. The degree of interaction and size/location of aggregates have a profound effect on the structure and physical properties of rennet- and acid-induced milk gels, and hence on cheeses.

2. AGENTS OF PROTEOLYSIS IN CHEESE

During ripening, proteolysis in cheese is catalysed by enzymes from (i) coagulant (e.g., chymosin, pepsin, microbial or plant proteinases), (ii) milk (plasmin and perhaps cathepsin D and other somatic cell proteinases), (iii) enzymes from the starter, (iv) nonstarter, or (v) secondary cultures (e.g., *P. camemberti*, *P. roqueforti*, *Propionibacterium* sp., *B. linens* and other coryneforms) and (vi) exogenous proteinases or peptidases, or both, used to accelerate ripening (Sousa *et al*., 2001).

2.1 MILK PROTEINASES

Milk contains several indigenous proteinases. Plasmin (fibrinolysin, EC 3.4.21.7) is the dominant indigenous milk proteinase and it has been the subject of many reviews (Grufferty and Fox, 1988; Bastian and Brown, 1996; Kelly and McSweeney, 2001). Plasmin is a component of blood, where its physiological role is solubilisation of fibrin clots. Moreover, plasmin is a component of a complex system consisting of the active enzyme, its zymogen (plasminogen), and activators and inhibitors of the enzyme. Plasmin is a trypsine-like serine proteinase with a pH optimum at about 7.5 and a high specificity for peptide bonds containing lysine at the terminal side. It is active on all caseins but especially on α_{s2}- and β-caseins (Fox *et al*., 2000). Milk contains low level of another indigenous acid proteinase: cathepsin D (EC 3.4.23.5). Cathepsin D is an aspartic proteinase with an optimum pH of 4.0 on haemoglobin and optimum temperature of 37°C (Kaminogawa and Yamauchi, 1972; Barrett, 1972). It is relatively heat labile (completely inactivated by 70°C x 10 min) and it has specificity similar to that of chymosin; however, its milk clotting potential has been reported to be very poor (McSweeney *et al*., 1995; Larsen *et al*., 1996). The level of cathepsin D present (around 0.4 mg/mL) in milk, in fact, is far too low to be of significance with respect to milk coagulation. In addition to cathepsin D, other proteolytic enzymes are present in milk, including thrombin, a lysine aminopeptidase, and poteinases from leucocytes, but they are considered not to be significant in cheese (Grufferty and Fox, 1998; Fox *et al*., 2000).

2.2 PROTEINASES FROM RENNET

Majority of cheeses produced around the world are manufactured traditionally, using an enzymatic coagulant extracted from the abomasa of milk-fed calves. This extract, known as calf

rennet, consists of two proteolytic enzymes: chymosin (EC 3.4.23.4), the major component (88–94% milk clotting activity, MCA) and bovine pepsin (EC 3.4.23.1; 6–12% MCA). The relative proportion of these enzymes varies with individuality and age of calves, the method of rennet separation and the conditions and pH values at which the milk clotting activity is measured (Guinee and Wilkinson, 1992). Chymosin is an aspartic proteinase which coagulates milk by specifically hydrolysing the Phe_{105}-Met_{106} bond in κ-casein. Most of the coagulant activity added to the milk is lost in the whey; only 0-15% of the rennet activity added to the milk remains in the curd after manufacture, depending on factors including type of coagulant, ratio of different enzymes in blends, cooking temperature, the cheese variety and the moisture level of the final cheese (Guinee and Wilkinson, 1992). Pepsins are more sensitive to denaturation by pH than chymosin and hence the amount of activity of these coagulants retained in the curd is very strongly dependent on the pH of the milk at setting and shortly thereafter (Fox and McSweeney, 1996). The heat stability of rennet at the temperature used during cooking of curds and whey also has a large effect on the level of rennet activity remaining in the curd.

2.3 PROTEINASES FROM PLANT COAGULANTS

The worldwide increase in cheese production along with the limited supply of calf rennet have led to an increase in the demand for alternatives sources of milk coagulants (Green, 1972; Cavalcanti *et al*., 2004). In this regard, plant proteinases have been investigated as source of milk-clotting enzymes, but only a small number of aspartic proteinases from plant origin have been isolated and partially characterised (Tavaria et al., 1997; Sousa, 1998). Many plant coagulant preparations were reported to have an excessively low ratio of milk clotting to proteolytic activity, which results in bitter peptides in ripened cheese, or to an excessively low clotting power that causes a low cheese yields. The difficulties experienced with these preparations result mainly from the unique composition of the plant extracts, which contain a complex cocktail of enzymes whose activity is difficult to control. An exception from the other plant proteinases is the proteinases from dried flowers of *Cynara cardunculus*, which have milk-clotting activity and have been employed successfully for many centuries in the Iberian Peninsula for the manufacture of traditional cheeses, e.g., Serra da Estrela (Roseiro, 1991; Macedo *et al*., 1993), La Serena (Nuñez *et al*., 1991; Roa et al., 1999), Guía (Fernández-Salguero *et al*., 1991) and Los Pedroches (Carmona *et al*., 1999; Fernández-Salguero and Sanjuán, 1999; Vioque *et al*., 2000). In the last years, the specificity of proteinases from *Cynara cardunculus* were studied in solutions of bovine (Faro *et al*., 1992; Sousa, 1993; Macedo *et al*., 1996) ovine and caprine caseins (Sousa and Malcata, 1998b), as well as primary proteolysis in cheeses manufactured from ovine milk and from ovine or caprine milk

(Sousa and Malcata, 1997a, b; 1998a; Sousa, 1998). Extracts from *C. cardunculus* were reported to contain two proteinases, cardosin A and cardosin B (Sousa, 1993; Veríssimo *et al.*, 1995; Veríssimo *et al*., 1996). Studies showed that cardosin A has a cleavage specificity similar to chymosin, whereas cardosin B resembles pepsin (Faro *et al*., 1992; Veríssimo *et al.*, 1995; Ramalho-Santos *et al.*, 1996).

2.4 PROTEOLYTIC ENZYMES FROM STARTER

The starter cultures commonly used in cheese manufacture include mesophilic *Lactococcus* and *Leuconostoc* species, thermophilic *Lactobacillus* species and *Streptococcus thermophilus*. The principal role of the starter culture is in the production of lactic acid, causing a decrease in pH. Although lactic acid bacteria (LAB) are weakly proteolytic, they possess proteinase and a wide range of peptidases, which are responsible for the formation of small peptides and amino acids in cheese (Fox *et al*., 2000; Sousa *et al*., 2001). The proteolytic system of *Lactococcus* has been studied thoroughly at the molecular, biochemical and genetic levels. The system of *Lactobacillus* spp. is less well characterized but the systems of both genera are generally similar. *Sc. thermophilus* is less proteolytic than *Lactococcus* or *Lactobacillus* and has been the subject of little research. The proteolytic systems of LAB have been extensively reviewed by many authors (Kunji *et al*., 1996; Law and Haandrikman, 1997, Monnet *et al*., 1993; Tan *et al*., 1993; Thomas and Pritchard, 1987 and Visser, 1993).

2.5 PROTEOLYTIC SYSTEM OF NON STARTER MICROFLORA

During the ripening of many cheeses, the starter lactococcal population declines and the initially small population of adventitious non-starter lactic acid bacteria (NSLAB) ultimately becomes the dominant bacterial population in the maturing cheese (Peterson and Marshall, 1990; Martley and Crow, 1993; Fox *et al*., 1998). The NSLAB microflora is dominated by a few species of mesophilic lactobacilli (*Lb. casei*, *Lb. paracasei*, *Lb. plantarum* and *Lb. curvatus*). Some authors have reported the presence of pediococci in cheese, but recent studies have failed to find them in significant numbers (Fox *et al*., 2000). NSLAB, although present initially at low numbers (<50 cfu/g), grow rapidly to reach ~10^7 cfu/g within 4 weeks and this number remains relatively constant thereafter (Folkertsma *et al*., 1996). The proteolytic activity of NSLAB appears to supplement that of the starter, producing peptides with generally similar molecular weights, and free amino acids (Lane and Fox, 1996; Lynch *et al*., 1997; Williams and Banks, 1997; Williams *et al*., 1998; Muehlenkamp-Ulate and Warthesen, 1999). Peptidolytic strains of NSLAB may therefore be considered for use as adjuncts in cheese-making both to manipulate the overall flavour profile of the

cheese and to accelerate the rate of flavour formation (Fox et al., 1998; Fox and Tobin, 1999; Madkor *et al*., 2000).

2.6 PROTEINASES FROM SECONDARY STARTER

Most cheese varieties have a secondary microflora. The principal secondary microflora is characterized by *Penicilium roqueforti* (Blue mould cheese), *Penicillium camemberti* (surface mould cheese, such as Camembert and Brie), *Brevibacterium linens*, *Propioni-bacterium freudenreichii* subsp. *shermanii* (Swiss-type cheese) and several species of yeasts (Fox *et al*., 2000). In many cheese varieties, secondary cultures are added intentionally and/or encouraged to grow by controlling environmental conditions. The surface microflora of smear-ripened cheese has two important functions: (i) production of enzymes (lipases, proteinases and peptidases) and (ii) deacidification of first the cheese surface and then the cheese body. For many years, *B. linens* have been known to be important bacteria growing on the surface of smear ripened cheeses; for this reason it is commonly used as ripening starter. *B. linens* secretes an extracellular proteinase and an aminopeptidase, possess a number of intracellular peptidases, which may be released on cell lysis (Rattray and Fox, 1997, 1999) and the specificity of the extracellular proteinases from *B. linens* on α_{s1}- and β-caseins has been determined (Fox *et al*., 1995; Rattray *et al*., 1996, 1997). *B. linens* is essential for smear cheese because of its aromatic and proteolytic properties and its bright orange pigments (Bockelmann *et al*., 1997a, b; Bockelmann *et al*., 1998). *P. roqueforti* and *P. camemberti* secrete aspartyl and metalloproteinases, which have been well characterised, including their specificity on α_{s1}- and β-caseins (Gripon, 1993). *Propionibacterium* spp. are weakly proteolytic but strongly peptidolytic and they are particularly active on proline-containing peptides during the ripening of Swiss-type cheeses, which may contribute to the characteristic flavour of these cheeses.

3. CATABOLISM OF AMINO ACIDS

Catabolism of free amino acids (FAA) can result in a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, all of which may contribute to cheese flavour (Fig.. The first stage in amino-acid catabolism involves decarboxylation, deamination, transamination, desulphuration or perhaps hydrolysis of the amino-acid side-chains. The second stage involves conversion of the resulting compounds (amines and α-ketoacids), as well as amino acids themselves, to aldehydes, primarily by the action of deaminases on amines. The final stage of amino-acid catabolism is the reduction of the aldehydes to alcohols, or their oxidation to acids. Sulphur-containing amino acids can undergo extensive conversion, leading to the formation of a number of compounds, including methanethiol and other sulphur derivatives. Amines, including

biogenic amines, are produced in cheese by enzymatic decarboxylation of FAA (Joosten and Stadhouders, 1987); the relative concentration of each amine depends on the cheese variety and the non-starter microflora. The principal amines in most cheeses are tyramine and histamine produced by decarboxylation of Tyr and His, respectively (Fox *et al*., 1995, Sieber and Lavanchy, 1990). Histamine when present at high levels can result in food poisoning (Santos, 1991), and some cases of histamine poisoning have occurred subsequent to the consumption of cheese (Taylor *et al*., 1982). No relationship has been found between the concentration of FAA and the production of amines in cheese (Smith, 1981), probably due to differences in the rates of decarboxylation of individual amino acids or in the rate of deamination of resulting amines (Polo *et al*., 1985). Simple decarboxylation can explain the formation of most amines found in cheese, but there is no readily available explanation for the formation of secondary and tertiary amines in cheese (Adda *et al*., 1982). On the other hand, deamination of FAA leads to the production of ammonia and α-keto acids (Hemme *et al*., 1982). α-Keto-3-methylbutanoic and α-keto-3- methylpentanoic acids have been reported to have an intense cheese-like odour (Muller *et al*., 1971), but the concentrations of these acids vary widely between cheeses. Ammonia is an important constituent in many cheeses such as Camembert, Gruyère and Comté (Fox *et al*., 1995), and *P. camemberti*, *G. candidum* and *Br. linen*s play a major role in ammonia production (Karahadian and Lindsay, 1987). Amines can be subjected to oxidative deamination, yielding aldehydes (Molimard and Spinnler, 1996). Enzyme-catalyzed transamination of FAA results in the formation of an intermediate imide that is subsequently degraded by decarboxylation or by the Strecker reaction (Molimard and Spinnler, 1996; Urbach, 1995), forming an aldehyde (Fox *et al*., 1995; Polo *et al*., 1985). At the beginning of cheese ripening, with a lower pH, amino acids are decarboxylated to amines. In the later stages of ripening when there is an increase in pH, these amines are subsequently oxidized to aldehydes via the Strecker degradation (Barbeiri *et al*., 1994). Aldehydes do not accumulate to high concentrations in cheese because they are rapidly transformed to alcohols or to the corresponding acids (Dunn and Lindsay, 1985; Lemieux and Simard, 1992). Phenylacetaldehyde, isobutanal, 3-methylbutanal and methional can be formed by this mechanism from Phe, Leu/Ile, Val and Met, respectively (Adda *et al*., 1982) ; while acetaldehyde can be derived from threonine with the help of a threonine aldolase, and ethanal can be produced by yeasts when alcohol dehydrogenase is less active than pyruvate decarboxylase (Molimard and Spinnler, 1996). Benzaldehyde may be produced from the α-oxidation of phenylacetaldehyde or from β-oxidation of cinnamic acid (Casey and Dobb, 1992; Sieber *et al*., 1995). Sulphur compounds are found in cheese must originate principally from methionine, as there are higher concentrations of Met in aseins than cysteine (Cys residues in the caseins are present at low levels only in α_{s2}- and κ-caseins). Low molecular weight sulphur

compounds in cheese include methanethiol (CH_3SH), hydrogen sulphide (H_2S), dimethylsulphide (DMS; CH_3SCH_3), dimethyldisulphide (DMDS; CH_3SSCH_3), dimethyltrisulphide (DMTS; CH_3SSSCH_3) and carbonyl sulphide (O = C = S). Sulphur compounds are thought to interact with each other and with other compounds in cheese, generating other volatile flavour compounds (Kim and Olson, 1989). Although the exact pathway for the non-enzymatic formation of CH_3SH has not been established, a mechanism proposed by Manning (1979a, b), in which a reducing agent produces H_2S from cystine/cysteine, which then reacts with methionine to produce methanethiol, is accepted by many investigators (Adda *et al*., 1985; Fox *et al*., 1995; Hemme *et al*., 1982). The concentration of H_2S in cheese increased during ripening, and initially no correlation was found between its concentration (or those of other sulphur compounds) and flavour development (Aston and Douglas, 1983), although Barlow *et al*. (Barlow *et al*., 1989) found a high correlation between the concentration of H_2S and flavour, particularly when the value for H_2S was combined with either the concentration of water-soluble nitrogen (WSN) or lactic acid. These authors suggested that these parameters (H_2S and WSN/lactate) were better predictors of the future quality of young cheeses than their flavour or composition at an early age. The presence of H_2S in fresh curd indicates that some may also be formed during pasteurization of cheese milk (Law *et al*., 1976). DMS, DMDS and DMTS are thought to be important contributors to cheese flavour (Barbeiri *et al*., 1994; Bosset and Liardon, 1985). DMS is a product of the metabolism of propionic acid bacteria formed from Met, and is a component of Swiss cheese flavour (Adda *et al*., 1982) . DMS and DMDS could be produced directly from methanethiol, but it is unclear how DMTS is produced in cheese. Several other volatile sulphur compounds have been identified in cheese. Methional, which can be produced by Strecker degradation (Aston and Dulley, 1982), contributes positively to cheese flavour when present at low concentrations, while at high levels, a sweet corn-like off-flavour is detectable (Barbeiri *et al*., 1994) . Methional, which has been reported to have the odour of raw potatoes (Muller *et al*., 1971). *S*-Methylthioacetate can be produced under certain conditions by strains of *Br. linens* (Ferchichi *et al*., 1986a,b). 3-Methylthiopropanal is presumably formed by transamination of methionine followed by decarboxylation of an intermediate imide (Barbeiri *et al*., 1994) . The concentration of carbonyl sulphide increases throughout ripening and is less variable than other sulphur compounds (Aston *et al*., 1983), but no correlation has been found between its concentration and cheese flavour. Catabolism of Trp produces indole pyruvate, which can be catabolised further to indole lactic acid, indole acetic acid, indole aldehyde and benzaldehyde (Gao *et al*., 1997; Hummel and Kula, 1984). Catabolism of Phe results in the formation of phenylpyruvate by the action of an aminotransferase (Gao *et al*., 1997). Phenylpyruvate, phenyl lactate and phenyl acetate have been reported to be detected as metabolites of lactococcal Phe

catabolism (Yvon *et al*., 1997). Also, nonenzymatic breakdown of phenylpyruvate to benzaldehyde and phenethanol has been reported (Kong, *et al*., 1996). Flavour compounds originating from Phe which have been identified in cheese (Adda *et al*., 1982; Dunn and Lindsay, 1985) or in model systems (Jollivet *et al*., 1992) include phenylmethanol, phenylethanol, phenylpropanone, methylphenyl hydroxyacetate, phenylacetaldehyde, phenylpyruvate and phenylethyl acetate. Catabolism of Tyr results in the formation of *p*-hydroxy-phenylpyruvate. Tyr serves as precursor for 3 compounds in cheese: tyramine formed by decarboxylation, and *p*-cresol and phenol formed by atypical Strecker degradation (Elsden *et al*., 1976). A major concern to the dairy industry is the occurrence of off-flavours. Compounds like *p*-cresol, phenethanol, phenylacetaldehyde and indole have been associated with these unclean flavours, which are believed to be formed via catabolism of aromatic amino acids. Tyramine is a biogenic amine that is frequently detected in cheese (Voight *et al*., 1978), and is the most common cause of monoamine intoxication. Monoamine intoxication is characterized by an increase in blood pressure, resulting in palpitations, severe headaches, hypertension, nausea and vomiting, and has been associated with the consumption of cheese (McCabe, 1986). Three different Asp catabolic pathways have been reported (Christensen *et al*.,1999); however, the details and distribution of these pathways are still not well understood. The pathways of Glu catabolism produce α-ketoglutarate by the action of an aminotransferase or dehydrogenase, or γ-aminobutyrate (GABA) by the action of a decarboxylase (Christensen *et al*.,1999). GABA production is known to occur in cheese, and Zoon and Allersma (Zoon and Allersma, 1996) have reported a correlation between the production of CO_2 and GABA in cheese with an increasing number of eyes. Thr catabolism converts Thr to acetaldehyde and Gly (Marshall and Cole, 1983); acetaldehyde is the flavour compound typical of yogurt flavour (Tamine and Deeth, 1980). Catabolism of branched-chain amino acids is most likely initiated in *L. lactis* by an aminotransferase, converting Leu, Ile, and Val to α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate, respectively. Catabolism of branched-chain amino acids is typically associated with the generation of compounds having a detrimental effect on product aroma or flavour in the production of fermented dairy products (Christensen *et al*.,1999). The aldehyde and alcohol products from catabolism of branched-chain amino acids are known to cause defects in cheese. Cheeses containing 18-90 ppm 3-methylbutanal and 9-45 ppm 3-methylbutanol, also produced by the catabolism of branched-chain amino acids, were graded lower for flavour quality and were maltier than the control cheeses (Braun and Olson, 1986) . Additionally, flavours described as harsh and dull were correlated with elevated concentrations of 3-methylbutanal and 2-methylpropanal (Dunn and Lindsay, 1985).

4. MATERIAL AND METHODS

4.1 STANDARD AND REAGENTS

Azocasein, trichloracetic acid (TCA), Bradford reagent, α_s-casein, β-casein, β-mercaptoethanol, glycine, trizma base, sodium dodecyl sulphate (SDS), glycerol, ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), low-molecular weight marker proteins, N,N'-methylene bis-acrylamide, and acrylamide (all electrophoretic grade) were supplied by Sigma Aldrich (Milan, Italy).

4.2 PLANT MATERIALS

The extract of *Cynara cardunculus* L. (CC) was purchased by Prodor (Bobbio, Piacenza, IT). To obtain a representative sample, latex was collected from *Ficus carica* L. trees, under the same environmental and soil characteristics, in the Puglia region (Foggia, Italy). All samples were harvested manually at the same stage of maturation (August 2009), by incising the stalk of the green fruit from the main branch. Several drops of the latex from the fig were allowed to drip into the test tubes. It was clarified by centrifugation at 14000g at 4°C for 15 min. The clear supernatant (CS) was separated from the gummy material (GM) and stored at -20°C until further use.

4.3 PROTEIN DETERMINATION

Protein concentration was determined by the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein.

4.4 PROTEINASE ACTIVITY

Proteinase activity was determined according to the method of Kilcawley *et al*. (2002), with some modifications. Enzyme solution (35 µg of protein) was added to a final volume of 600 µL of different buffers (pH 3.0-6.0). Azocasein substrate (0.5%) was prepared in Milli-Q water and added to the enzyme solution to the final volume of 1 mL. The same mixture of reagents without enzyme and heat-inactivated enzyme was used as blank and control, respectively. After incubation for 30 min at 37°C, the reaction was stopped by addition of 100 µL of 2 M TCA, mixed and centrifuged at 10000 *g* for 5 min. A sample (750 µL) of supernatant was transferred to a cuvette to which 250 µL of 0.5 M NaOH were added. The dye of the sample was measured spectrophotometrically at 440 nm, using a Varian Cary 50 Bio UV/VIS spectrophotometer. The proteinase activity was defined as the increase in absorbance units per minute (Δabs/min) and expressed as arbitrary units on µg of protein (AU/ µg protein/30 min); 1AU = 0.001 Δabs/min. All the experiments were performed in triplicate.

4.4.1 EFFECT OF pH AND TEMPERATURE ON PROTEINASE ACTIVITY

The effect of pH on proteinase activity was determined at 37°C within the range of pH 3.0-10.0. The buffers used were 50 mM Na-acetate (pH 3.0-5.0), 50 mM Na-phosphate (pH 6.0-7.0), 50 mM Tris-HCl (pH 8.0-9.0), 50 mM Na-carbonate (pH 10.0). Similarly, an analysis of the effect of temperature on the proteinase activity of the plant enzymes was carried out to determine the temperature optimum. Enzyme samples were incubated at different temperatures in the range of 20-90°C at their optimal pH. Activity measurements were performed as described above.

.

4.5 HYDROLYSIS OF BOVINE CASEIN

Commercial bovine α_s-casein (α_s-CN, α_{s1}-CN with a small proportion of α_{s2}-CN) and β-casein (β-CN) were dissolved up to 2 mg mL^{-1} in 100 mM sodium phosphate buffer (PBS) pH 6.5. *Cynara cardunculus* L. extract (CC) and the clear supernatant (CS) of *Ficus carica* L. latex were added (3 and 30 µg of protein) to each casein solution and hydrolyses were carried out at 37°C in a thermostated reaction vessel. At defined time intervals from 5 to 90 min, aliquots of α-CN and β-CN hydrolysates were removed and heated at 100°C for 10 min to stop proteolytic reaction. Samples were stored at -20°C until further analyses.

4.6 ELECTROPHORETIC CHARACTERIZATION OF HYDROLYSATES

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to examine the time-dependent proteolysis of bovine caseins by plant extracts. SDS-PAGE was performed in a vertical gel apparatus Mini-Protean II (Bio-Rad Laboratories, Milan, Italy) on 18% (w/v) according to the procedure of Laemmli (1970). Hydrolysate samples containing approximately 10 µg of protein were treated with loading buffer (0.5 M Tris-HCl pH 6.8, 20% glycerol, 4.4% SDS, 10 mg/mL bromophenol blue and 300 mM β-mercaptoethanol) and heated at 100 °C for 5 min. then boiled for 5 min before SDS-PAGE. Electrophoresis was run at 100 V until the tracking dye bromophenol blue disappeared from the separating gel. After electrophoretic separation, the gels were stained with 2 g Coomassie brilliant blue R-250 (in a 10:40:50 solution of acetic acid: methanol: water) and de-stained with a 10:40:50 solution of acetic acid: methanol: water. The molecular mass standards (Bio-Rad Laboratories, Milan, Italy) were bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), bovine carbonic anhydrase (29.0 kDa), bovine trypsinogen (24.0 kDa), trypsin inhibitor (20.0 kDa); bovine α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

4.7 Densitometric Analysis

Analysis of each electrophoresis was performed by capturing the image of each gel using a scanner (Gel Logic 112 Imaging System) and saving it as a graphic file in TIF format. Densitometric analysis was performed by Carestream Molecular Imaging Software (Mac & PC) v5.0.2. Quantification was based on the measurement of the areas of each peak of α-casein and β-casein or their generated peptide fragments as relative percentages of the total casein.

4.8 Gelatin SDS-PAGE

Enzyme activities of plant extracts were detected by zymography. A volume of 40 µL of each extract was loaded into SDS-PAGE gels containing 0.1% gelatin (w/v). Electrophoresis (SDS-PAGE) was performed with a 4.9% (w/v) polyacrylamide stacking gel in 0.125M Tris-HCl buffer, pH 6.8 and with a 10% (w/v) polyacrylamide resolving gel in 0.38M Tris-HCl buffer, pH 8.8 containing 0.1% (w/v) SDS, for 220 min at 80 V. After electrophoretic migration, the gels were washed three times in tris buffer saline (TBS) pH 7.2 for 30 min. The hydrolysis reaction then proceeded inside the gel during incubation at 37°C for 24 h. The active enzymes were revealed as translucent bands after incubation of the gels, first in Coomassie Brilliant Blue for 60 min, and second in a destaining solution.

4.9 Statistical Analysis

ORAC data are presented as means ± SD. Statistical analysis was carried out using statistical package Statgraphics Centurion XVI (StatPoint Technologies Inc., USA, 2010) program and significance of each group was verified with the analysis of the One-way ANOVA followed by the Tuckey test at $p < 0.05$.

5. Results and Discussion

Proteinases, also called as proteolytic enzymes or proteinases, refer to a group of enzymes whose catalytic function is to hydrolyze peptide bond of proteins. Proteinases are the most commercially important enzymes due to their multiple applications in the food, pharmaceutical and detergent industries (Doran, 2002). One of the main applications of proteinases in the food industry is the use of rennet in cheese making. Milk-clotting enzymes have been found in almost all kinds of plant tissues and it appears to be a general rule that all proteolytic enzymes possess the ability to clot milk under proper conditions (Tamer and Mavituna, 1997). In this regard, plant proteinases, i.e., *Cynara cardunculus* L. and *Ficus carica* L., could stimulate an interest in local cheese production and be a valid alternative to the traditional calf rennet. The objective of the current study

was (i) to characterize the biochemical properties (pH and temperature) of proteinases from *Cynara cardunculus* L. (CC) and *Ficus carica* L. (CS) in order to assess their potential use in cheese making, and (ii) to evaluate the effect of their enzyme concentration on the profile of hydrolysis of bovine α_s- and β-caseins, which can be fruitful as source of biologically active peptides. Several plant proteinases have been purified and characterized by many authors (Lo Piero *et al*., 2002; Bruno *et al*., 2002; Sidrach *et al*., 2004; Devaraj *et al*., 2008; Azarkan *et al*., 2011). In this study, however, *Cynara cardunculus* L. and *Ficus carica* L. proteinases were not isolated; thus crude extracts were employed for the experiments. Although the properties of the crude enzyme preparation may well be derived from a combination of proteins, characterization of crude extracts is regarded as essential due to their potential use in cheese technology (Chazarra *et al*., 2007).

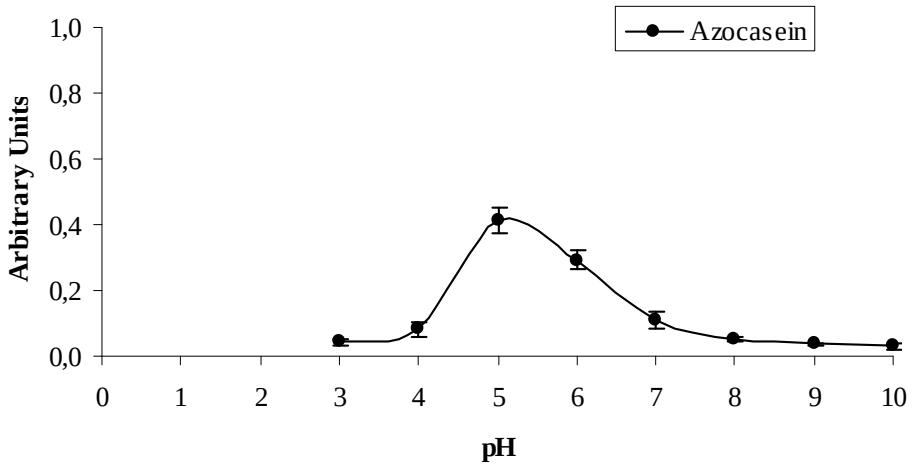

Fig. 1 Effect of pH on *Cynara cardunculus* L. (CC) proteinase activity. Proteolytic activity was measured using azocasein as substrate.

In addition to being costly, the overall process of purification is rather slow and cumbersome; hence, it is hardly appropriate when large quantities of those enzymes are sought (Silva *et al*., 2002). The enzymatic action of crude extracts of *Cynara cardunculus* L. and *Ficus carica* L. was firstly spectrophotometrically characterized *in vitro* at different pHs and temperatures. Several colourimetric methods have been described to detect and quantify proteinase activity (Rollema *et al*., 1989). Some of them measure the degradation of natural milk substrates due to the action of the proteolytic enzymes through the determination of the concentration of acid-soluble amino acids and peptides. However, there are other types of assays which specifically and directly measure the proteolytic activity of the samples by using synthetic substrate which, when hydrolyzed, releases coloured products. Azocasein (Charney and Tomarelli, 1947; Christen and Marshall; 1984) is one of these substrates; thus it was employed in the biochemical characterization. At first, proteolytic

activity was studied in terms of pH optimum in order to identify the best buffered conditions of these enzymes. The pH-activity profile of *Cynara cardunculus* L. (CC) crude extract is shown in Fig. 1. The plant extract exhibited optimum activity at pH 5.0 (Fig. 1). This result seems to support the concept that, as the optimum pH falls within the range of the pH acid, CC is an aspartic proteinase. The pH optima of aspartic proteinases, in fact, normally lies between 1.5 and 5.0. In the case of *Cynara cardunculus* L. crude extract, the optimum pH was similar to that found by other authors (Heimgartner *et al*., 1990; Llorente *et al*., 2004; Sidrach *et al*., 2005). On the contrary, *Ficus carica* latex exhibited a broad pH activity profile (Fig. 2). The maximum activity was observed between pH 6.0 and 9.0, and its optimum was shown at pH 7.0., revealing that the enzyme was more active in the neutral range. The lowest activity was detected in the acidic range, indicating the liable nature of ficin in acidic conditions. Similar findings were reported earlier by Kramer and Whitaker (1964), who showed that the different ficin fractions from *Ficus carica* were more active in the neutral pH range, while Englund *et al*. (1968) remarked the acidliable nature of ficin from *Ficus glabrata*.

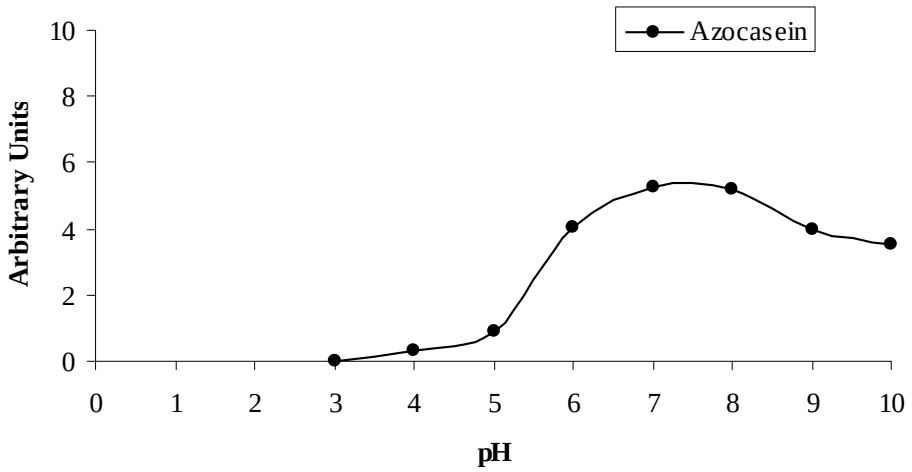

Fig. 17 Effect of pH on *Ficus carica* L. (CS) proteinase activity. Proteolytic activity was measured using azocasein as substrate.

Recently, Devaraj *et al*. (2008) observed a similar pH-profile for a purified ficin from *Ficus carica* latex. Finally, to study the influence of temperature on proteolytic activity of these plant extracts, a temperature range from 20 to 90°C was selected. Fig. 3 and 4 show that the proteolytic activity both for cardosin and ficin was optimal at 60°C, using azocasein as substrate. However, at higher temperatures there was a rapid decline in the enzymatic activity.

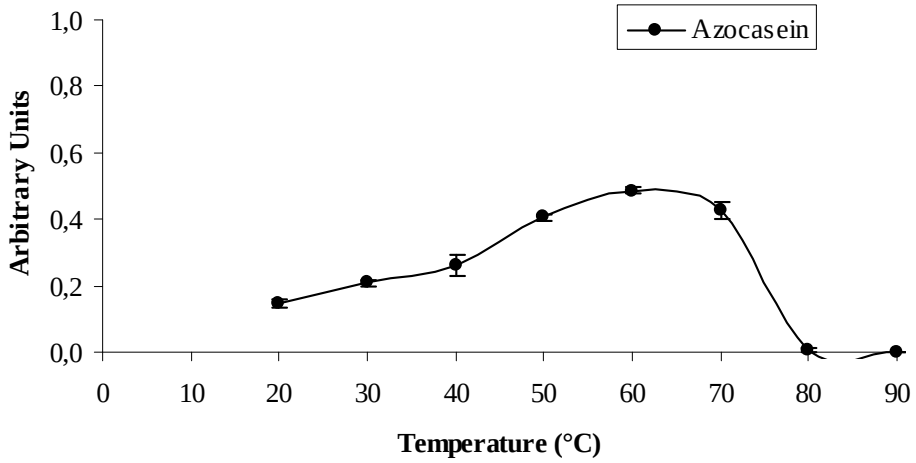

Fig. 3 Effect of temperature on *Cynara cardunculus* L. (CC) proteinase activity. Proteolytic activity was measured using azocasein as substrate.

The thermophilic nature of other plant proteinases was reported differently by Sidrach *et al*. (2005) and Chazarra *et al*. (2007) on cynarase (70°C), Raposo and Domingos (2008) on the proteinase of *Centaurea calcitrapa* (52°C), Lo Piero *et al*. (2002) on the lettucine from *Lactuca sativa* (50°C).

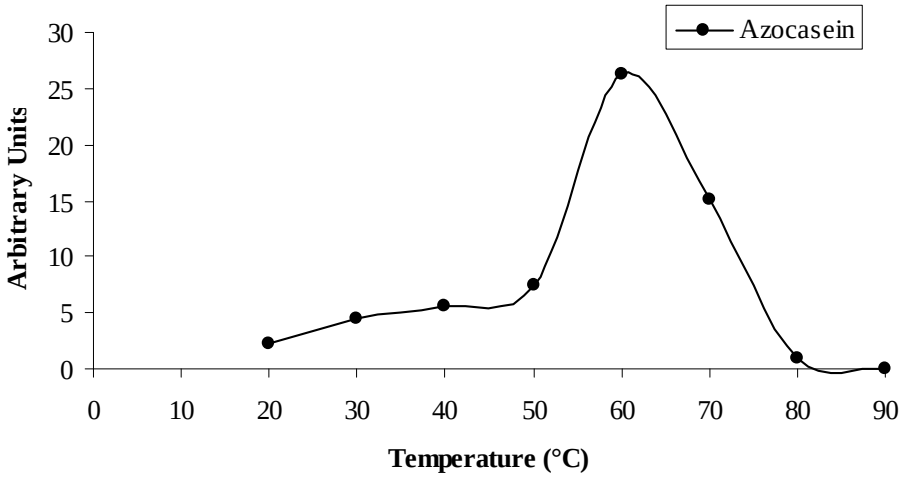

Fig. 4 Effect of temperature on *Ficus carica* L. (CS) proteinase activity. Proteolytic activity was measured using azocasein as substrate.

Nevertheless, these results are comparable to earlier reports. Yadav *et al*. (2006) characterized a serine proteinase from *Euphorbia milii* with a maximum activity around 60°C. A similar optimum temperature was showed by *Ficus racemosa* (Devaraj *et al*., 2008). Overall, such pH and temperature profiles can make these plant proteinases excellent enzymes for dairy industry. Furthermore, *Ficus carica* latex exhibited a higher and broader proteolytic activity than the crude

extract of *Cynara cardunculus*. This different pattern could be related to the proteinase specificity of these plant extracts. Therefore, it is very important for cheese manufacture to minimize the general non-specific proteolysis and prevents excessive proteolysis during ripening, ensuring the correct ratio between protein and peptides (Duarte *et al*., 2009). Hence, when a potential rennet substitute is studied, it is important to evaluate the degradation patterns of the caseins because of their effect on the yield, texture, and flavor of the final cheese (Fox, 1989). This purpose was achieved by assessing the hydrolysis of α_s- and β-caseins by *Cynara cardunculus* and *Ficus carica* qualitatively and quantitatively. Proteolysis of bovine caseins by the crude plant extracts was characterized qualitatively by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique; while quantification of intact casein fractions was examined using densitometry. At this purpose, experimental conditions similar that parallel milk (pH 6.5) were employed to monitor the evolution with time of the breakdown patterns of bovine caseins. Furthermore, a comparison of both crude extracts was carried out at two different enzyme concentrations (3 and 30 µg), as preliminary study of casein breakdown in model systems. As expected, the extent of degradation of α_s- and β-caseins by both plant extracts depended on the dose of enzyme used as well as the time of hydrolysis. The proteolytic activity, in fact, increased rapidly at higher enzyme concentration, producing a major hydrolysis as time elapsed (Fig 5, 6, 7, 8). In particular, *Cynara cardunculus* and *Ficus carica* showed to hydrolyze α_s-casein faster than β-casein. Therefore, the bonds in α_s-casein appeared to be more susceptible to degradation than β-casein. This observation is consistent with published data encompassing actual cheeses manufactured with extracts of *Cynara cardunculus* as rennet (Carretero *et al*., 1994; Mora and Marcos, 1981; Mulvihill and Fox, 1977; Sousa and Malcata, 1996; Tavaria *et al*., 2001; Silva *et al*., 2002). The hydrophobic nature of β-casein probably made it more resistant to the hydrolysis than α_s-casein. When cardosin was employed as proteinase, an extensive degradation of α_s-CN occurred by 15 and 5 min at the enzyme concentration of 3 and 30 µg, respectively. Furthermore, this casein fraction was hydrolyzed to a set of bands of higher electrophoretic mobility and poorly stained at the bottom of the gel, which may correspond to short and medium peptides released during the catalytic reaction (Fig. 5a, b). A similar pattern was observed in solutions of bovine α_s-casein incubated with extract from flowers of *Cynara cardunculus* (Sousa, 1993). Other authors (Sousa and Malcata, 1998b), who employed aqueous extract of *Cynara cardunculus* flowers on ovine and caprine Na-caseinate at 30°C at pH 6.5, obtained the same bands. Also *Ficus carica* latex showed to hydrolyze extensively and completely α_s-casein. In particular, 3 µg of ficin broke down α_s-casein in 5 min of enzymatic reaction and no bands were detected after this time of hydrolysis (Fig. 6a, lanes 4-9). However, the proteolytic activity of ficin was higher with 30 µg of the catalytic protein, leading to the immediate

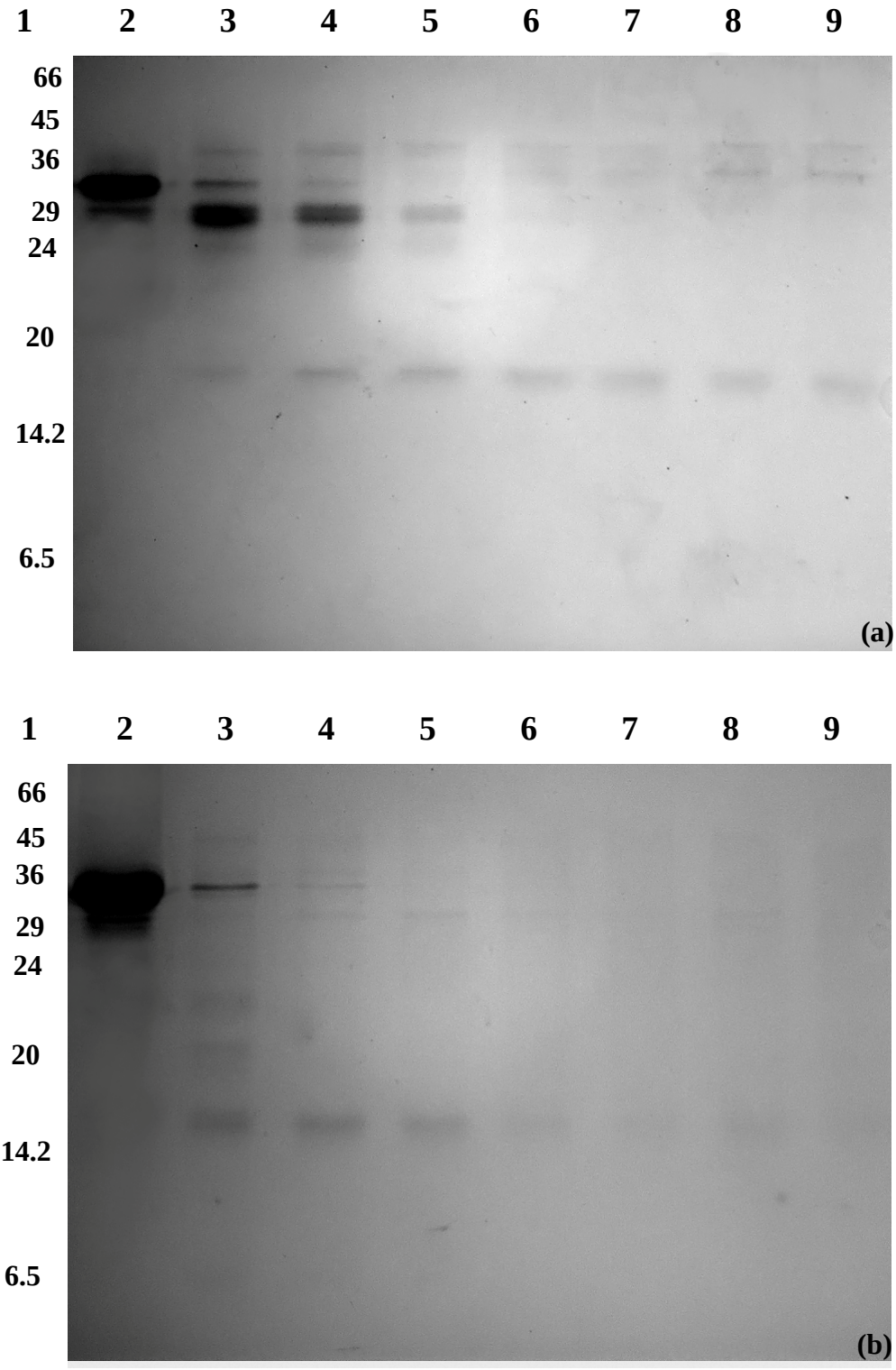

Fig. 518 SDS-PAGE electrophoretograms illustrating the degradation of bovine α_s-casein by the crude extract of *Cynara cardunculus* (CC) at (*a*) 3 µg and (*b*) 30 µg of enzyme concentration. Lane 1 contains molecular weight proteins markers (the same for the two gels). Lanes 2 contains the intact α-casein. Lanes 3-9 correspond to the hydrolysis of α-casein at different times of incubation: (3) 5 min; (4) 15 min; (5) 30 min; (6) 45 min; (7) 60 min; (8) 75 min; (9) 90 min.

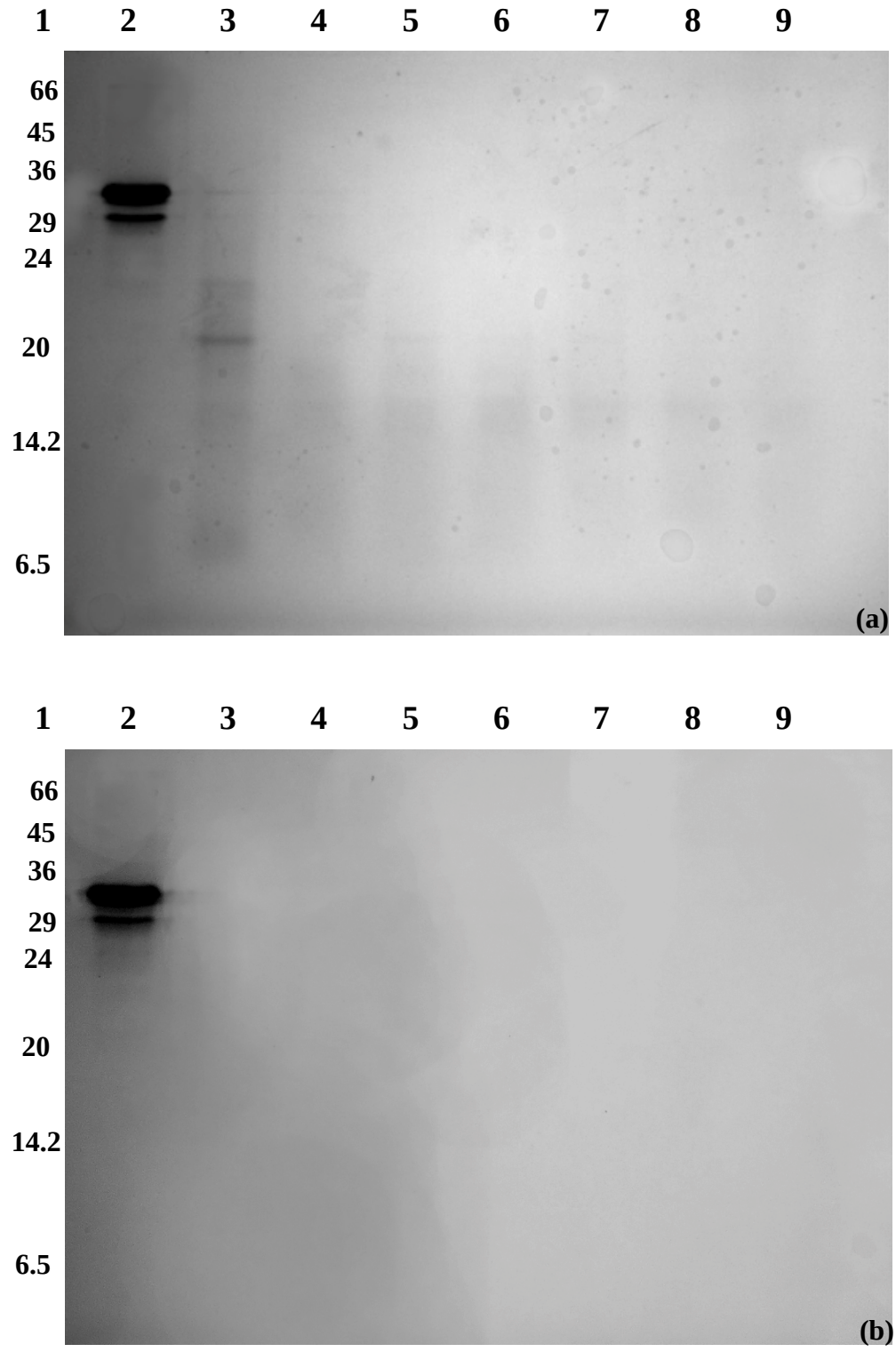

Fig. 6 SDS-PAGE electrophoretograms illustrating the degradation of bovine α-casein by the crude extract of *Ficus carica* (CS) at (*a*) 3 µg and (*b*) 30 µg of enzyme concentration. Lane 1 contains molecular weight proteins markers (the same for the two gels). Lanes 2 contains the intact α-casein. Lanes 3-9 correspond to the hydrolysis of α-casein at different times of incubation: (3) 5 min; (4) 15 min; (5) 30 min; (6) 45 min; (7) 60 min; (8) 75 min; (9) 90 min.

hydrolysis of α_s-casein within 5 min (Fig. 6b). On the contrary, it was noteworthy that β-CN was less sensitive to the action of both proteinases than α_s-CN (Fig. 7, 8). Bovine β-casein was still present within 90 min of hydrolysis with 3 µg of cardosin (Fig. 7a). In the specific, the breakdown of this casein fraction yielded four bands in 5 min of hydrolysis. At 15 min, however, the first three bands markedly disappeared, whereas traces of the fourth one still remained and seemed to vanish as time elapsed. Simultaneously a new band appeared (molecular weight ca. > 20 kDa), increasing its intensity gradually. A different pattern of hydrolysis and a major number of bands were observed when β-casein was incubated with 30 µg of cardosin. In addition to the main two bands detectable with a low concentration of the same crude extract, other bands with lower molecular weight (ca. < 20 kDa) came out within 60 min (Fig. 7b, lanes 2-6). Also in this case new peptides appeared as a consequence of caseins hydrolysis, detectable by a set of bands of higher electrophoretic mobility which became more intense as reaction time elapsed (Fig. 7b, lanes 3-9).

On the other hand, the hydrolysis of β-casein with 3 µg of ficin yielded five major bands, which remained intact for up to 45 min (Fig. 8a, lanes 2-6). Besides, a higher dose of ficin (30 µg) determined an extensive hydrolysis of β-casein within 5 min (Fig. 8b). These general observations were confirmed by the densitometric analysis, which allowed to quantify the rate of hydrolysis of caseins for each enzyme concentration used. The kinetics of caseins digestion by *Cynara cardunculus* and *Ficus carica*, expressed as relative percentages of the total casein, are shown in Fig. 9-10-11-12-13. Bovine α_s-CN was hydrolyzed by cardosin within 30 min at both concentrations: the residual amount of α_s-CN within 30 min was 10.75 and 5.2% at 3 and 30 µg, respectively (Fig. 9). This result remarked the effect of the concentration on the rate of hydrolysis. On the other hand, 3 µg of *Cynara cardunculus* degraded up β-CN to 40.51% within 5 min; while the residual β-CN was 11.79% at the end of enzymatic reaction (90 min) (Fig. 10). A different proteolysis pattern was noted with the increase of the enzyme concentration up to 30 µg: β-CN decreased by 94.9% after 60 min of incubation. As described previously, β-CN was also hydrolyzed to a set of bands of higher electrophoretic mobility at the bottom of the gel, whose kinetic is shown in Fig. 11. Contrary to the other densitograms, the kinetic and the intensity of these bands increased as time elapsed. This degradation pattern could correspond to short and medium peptides released during the catalytic reaction. The generation of these peptides was approximately of 12.63 and 60.81% at 5 and 90 min of incubation, respectively. Therefore, this result could encourage the research on peptides with biologically activity. Also the densitograms of ficin confirmed the previous observations (Fig. 12-13). *Ficus carica* latex did not show a gradual proteolytic action on bovine caseins. In particular, α-casein was degraded up to 11.27% in 5 min of hydrolysis with an enzyme dose of 3 µg, whereas no residual casein was quantified within 5 min at the increased

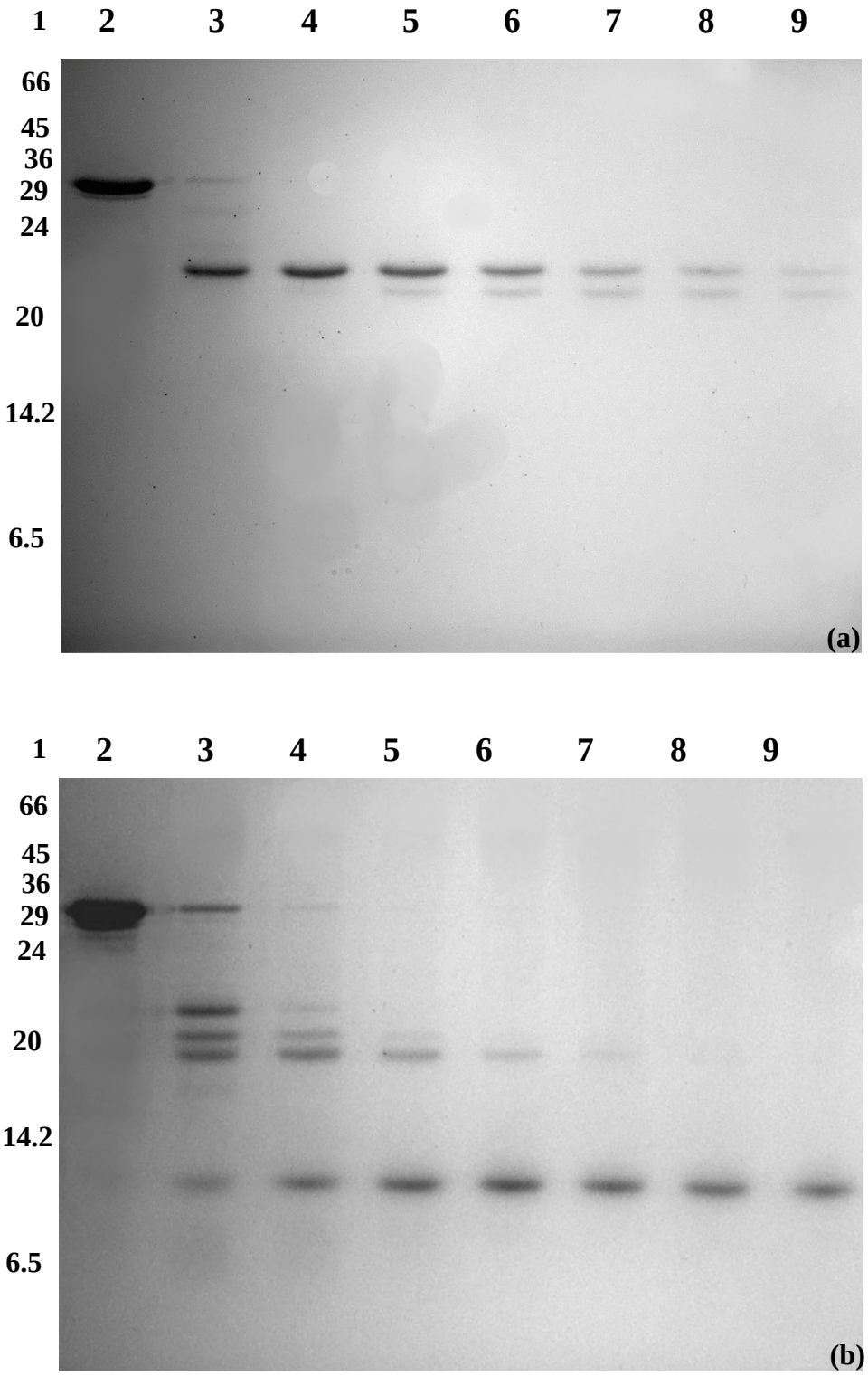

Fig. 7 SDS-PAGE electrophoretograms illustrating the degradation of bovine β-casein by the crude extract of *Cynara cardunculus* (CC) at (*a*) 3 µg and (*b*) 30 µg of enzyme concentration. Lane 1 contains molecular weight proteins markers (the same for the two gels). Lanes 2 contains the intact β-casein. Lanes 3-9 correspond to the hydrolysis of α-casein at different times of incubation: (3) 5 min; (4) 15 min; (5) 30 min; (6) 45 min; (7) 60 min; (8) 75 min; (9) 90 min.

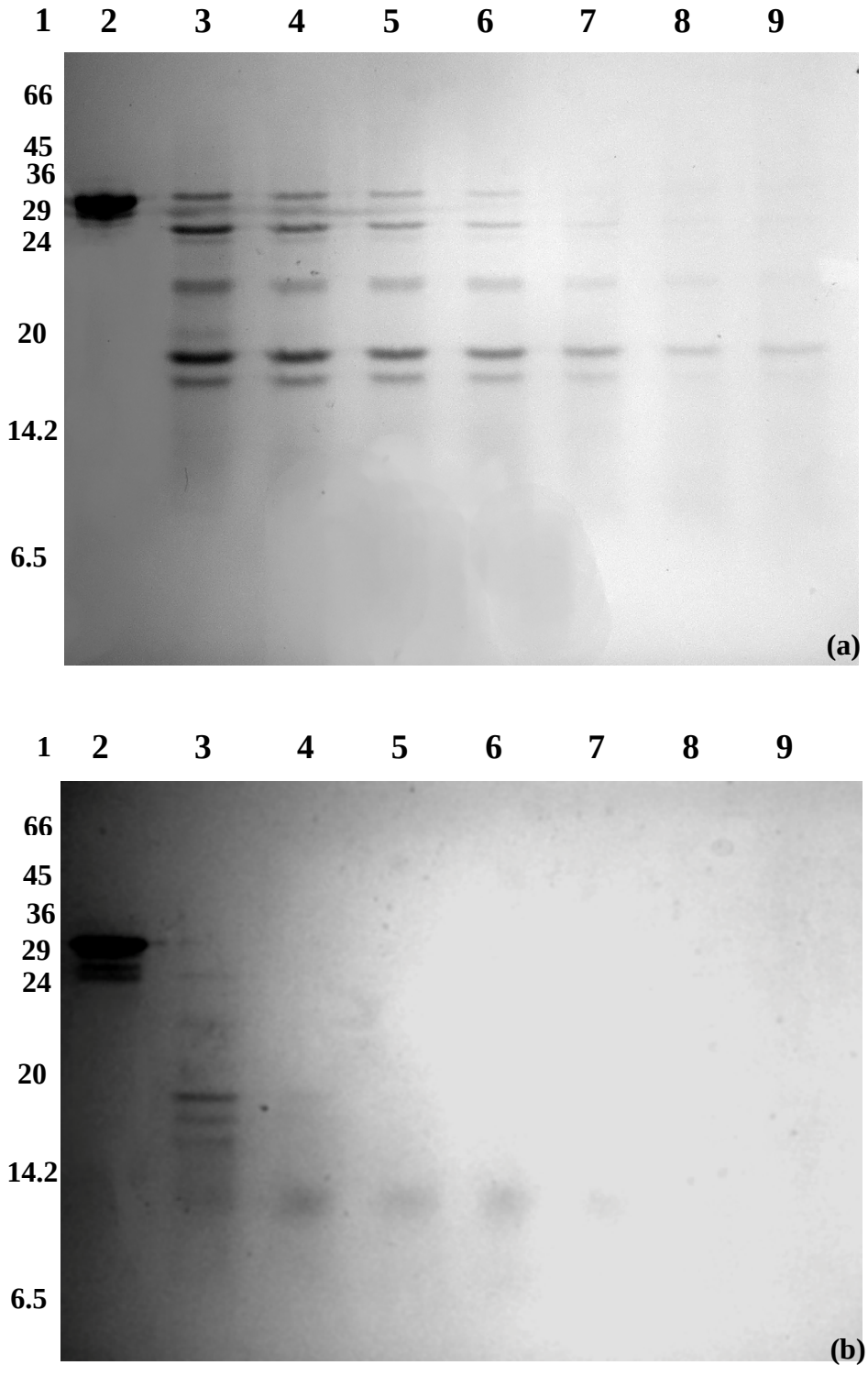

Fig. 8 SDS-PAGE electrophoretograms illustrating the degradation of bovine β-casein by the crude extract of *Ficus carica* (CS) at (*a*) 3 µg and (*b*) 30 µg of enzyme concentration. Lane 1 contains molecular weight proteins markers (the same for the two gels). Lanes 2 contains the intact β-casein. Lanes 3-9 correspond to the hydrolysis of α-casein at different times of incubation: (3) 5 min; (4) 15 min; (5) 30 min; (6) 45 min; (7) 60 min; (8) 75 min; (9) 90 min.

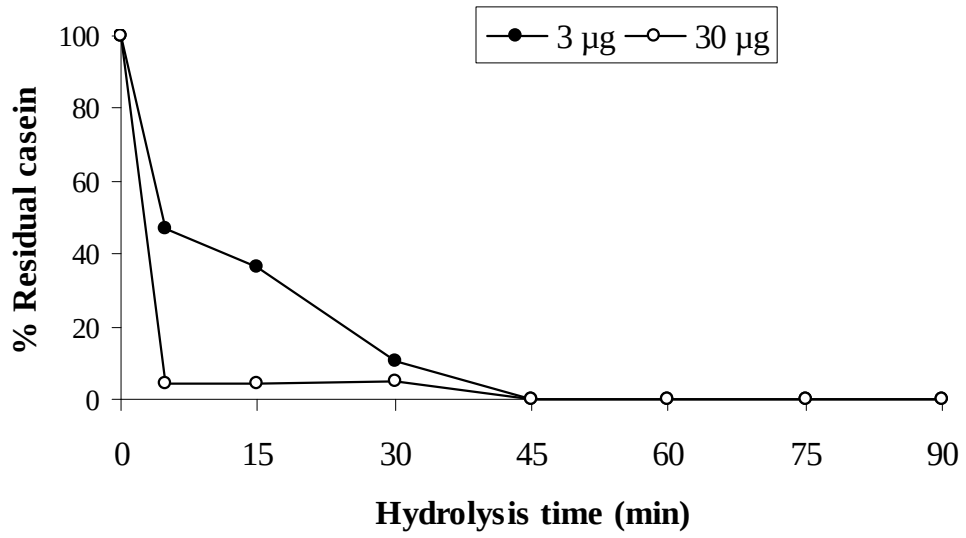

Fig. 9 Degradation of α_s-casein by *Cynara cardunculus* at 3 and 30 µg (,pH 6.5, 37°C). Time-course of digestion: 5 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. Values come from the corresponding densitograms of SDS-PAGE.

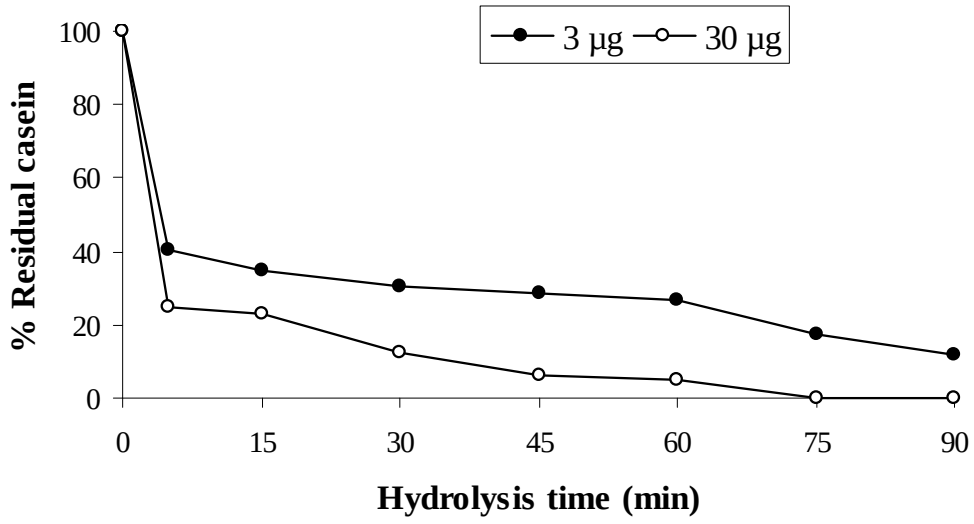

Fig. 1019 Degradation of β-casein by *Cynara cardunculus* at 3 and 30 µg (pH 6.5, 37°C). Time-course of digestion: 5 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. Values come from the corresponding densitograms of SDS-PAGE.

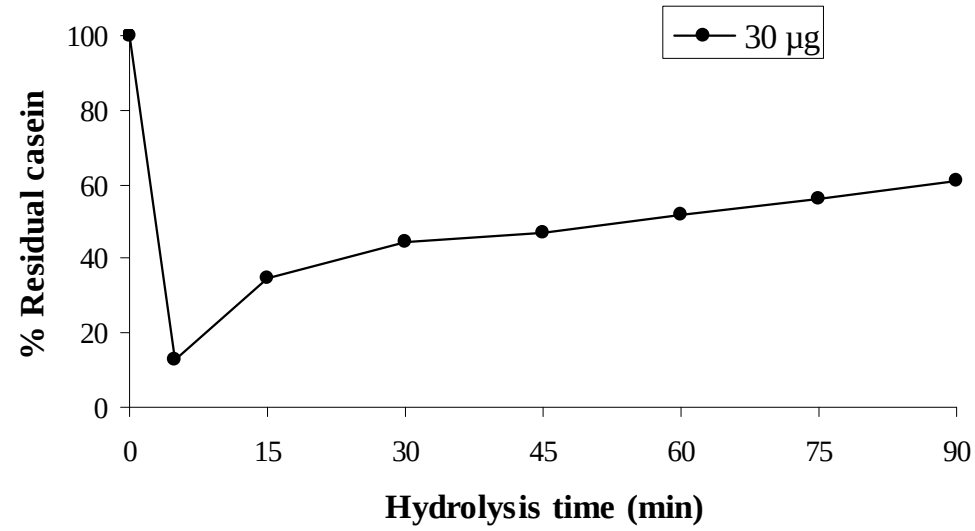

Fig. 11 Degradation of β-casein by *Cynara cardunculus* (30 µg, pH 6.5, 37°C). Time-course of digestion: 5 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. Values come from he corresponding densitograms of SDS-PAGE.

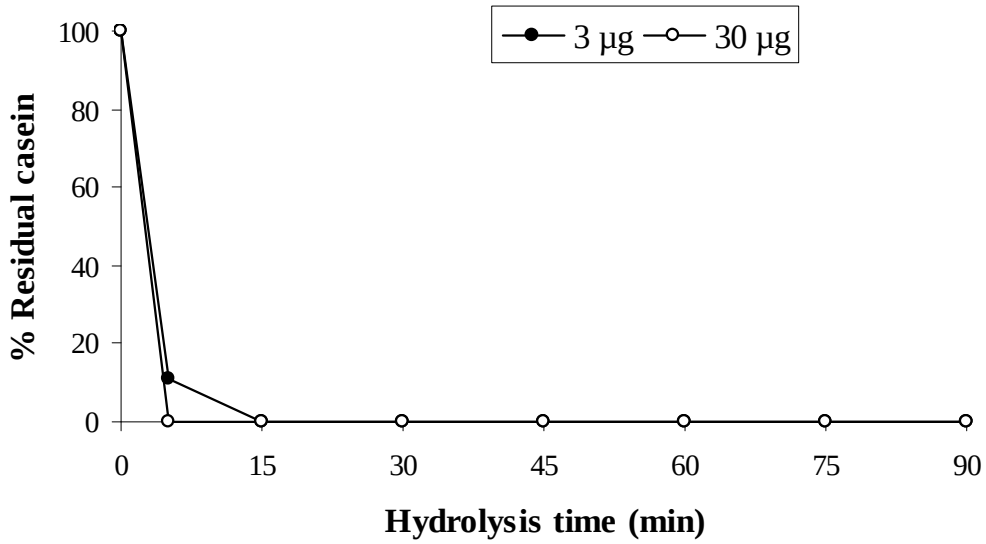

Fig. 12 Degradation of α_s-casein by *Ficus carica* at 3 and 30 µg (pH 6.5, 37°C). Time-course of digestion: 5 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. Values come from the corresponding densitograms of SDS-PAGE.

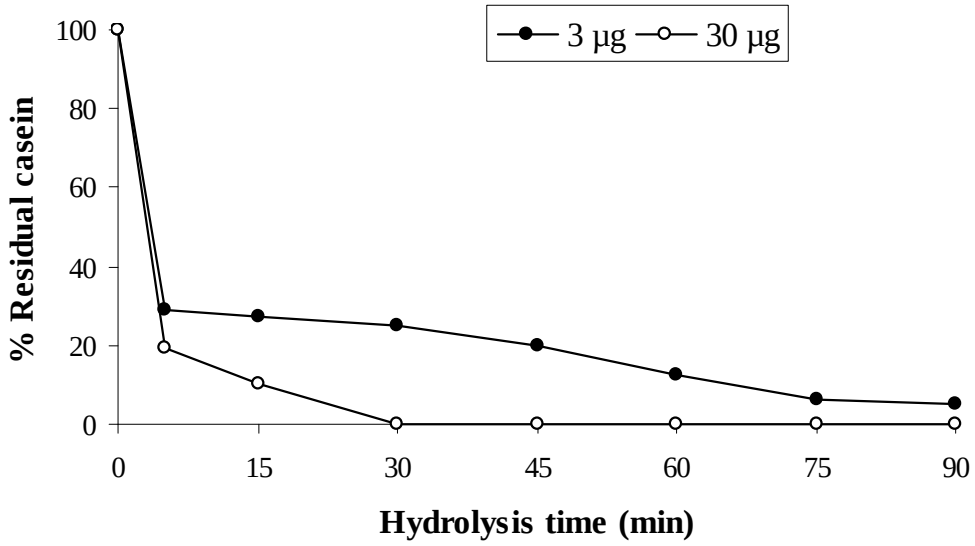

Fig. 13 Degradation of β-casein by *Ficus carica* at 3 and 30 µg (pH 6.5, 37°C). Time-course of digestion: 5 min, 15 min, 30 min, 45 min, 60 min, 75 min and 90 min. Values come from the corresponding densitograms of SDS-PAGE.

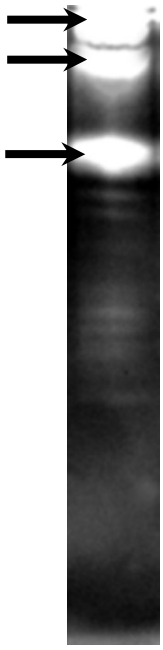

Fig. 1420 Gelatin zymogram analysis performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *Ficus carica* latex (CS).

enzyme concentration (Fig. 11). Also ficin showed to hydrolyze slowly and gradually β-casein with the dose of 3 µg. In comparison to the first 5 min of hydrolysis, β-CN was digested up to ca. 9 and 23.6% at 45 and 90 min, respectively. On the contrary, the digestion of β-CN with 30 µg of ficin proceeded fastly, reporting ca. 10% of residual casein at 15 min (Fig. 13). From these results, it was concluded that both the source of proteolytic enzymes and

the nature of the substrates yielded significant effects in the percent degradation of bovine caseins. As observed with the azocasein assay, *Cynara cardunculus* exhibited a specific and lower proteolytic activity than *Ficus carica*. This result agrees with that of Nouani *et al.* (2009), who found that the proteolytic activity of *Ficus carica* had a more marked kinetic on casein compared to *Cynara scolymus*, another variety belonging to the family of Asteraceae. To better study the broad specificity of *Ficus carica* proteinase, zymogram in denaturing conditions was carried out.

Zymography is a simple, sensitive, and quantifiable technique that is widely used to detect functional enzymes following electrophoretic separation in gels (Leber and Balkwill, 1997; Kim *et al.*, 1998). Such methods use sodium dodecyl sulfate (SDS)–polyacrylamide gels, which are copolymerized with a substrate degraded by the enzymes during incubation after electrophoretic separation. Activity in zymograms is visualized as clear bands, and at this purpose, gelatin was used as substrate to detect enzymatic activity of *Ficus carica* latex. Gelatin zymography, in fact, has the advantage of distinguishing different species of enzymes due to their mobility difference (Raser *et al.*, 2005). The zymogram of *Ficus carica* proteinase towards gelatin displayed three major proteolytic bands and other ones less intense (Fig.14). Moreover, ficin activity was detected mainly in the stacking portion of the gel, indicating that the enzyme aggregated extensively. Therefore, the enzymatic activity of this plant proteinase was the expression of many isoforms electrophoretic mobility, indicating a high level of polymorphism.

6. CONCLUSIONS

In summary, this chapter reports the proteolytic activity of *Cynara cardunculus* L. and *Ficus carica* L. crude extracts on bovine casein. The biochemical properties of these proteinases, i.e., pH and temperature, are useful for exploring the potentials applications of these plant extracts in dairy industry. The proteolytic activity of both plant crude extracts appears to be related to the type and specificity of proteinase, enzyme concentration, type of substrate as well as time of hydrolysis. Moreover, the densitometric analysis is a useful technique to quantify proteolytic activity. Further studies need to be carried out to assess the use of *Cynara cardunculus* and *Ficus carica* proteinases in cheese making and their contribution on proteolysis, development of flavour as well source of bioactive peptides.

7. REFERENCES

Adda, J., Gripon, J. C. and Vassal, L. (1982) 'The chemistry of flavour and texture generation in cheese', *Food Chemistry*, 9, 115-129.

Aston, J. W. and Dulley, J. R. (1982) 'Cheddar cheese flavour', *Australian Journal of Dairy Technology*, 37, 59-64.

Aston, J. W. and Douglas, K. (1983) 'The production of volatile sulphur compounds in Cheddar cheeses during accelerated ripening', *Australian Journal of Dairy Technology*, 38, 66-70.

Aston, J. W., Greive, P. A., Durward, I. G. and Dulle, J. R., (1983) 'Proteolysis and flavour development in Cheddar cheese', *Australian Journal of Dairy Technology*, 38, 59-65.

Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J. and Baeyens-Volant, D. (2011) 'Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from *Ficus carica* latex', *Phytochemistry*, 72, 1718-1731.

Barbeiri, G., Bolzoni, L., Careri, M., Mangia, A., Parolari, G., Spagnoli, S. and Virgili, R. (1994) 'Study of the volatile fraction of Parmesan cheese', *Journal of Agricultural and Food Chemistry*, 42, 1170-1176.

Barlow, I., Lloyd, G. T., Ramshaw, E. H., Miller, A. J., McCabe, G. P. and McCabe, L. (1989) 'Correlations and changes in flavour and chemical parameters of Cheddar cheeses during maturation', *Australian Journal of Dairy Technology*, 44, 7-18.

Barrett, A. J. (1972) 'Lysosomal enzymes', in Dingle, J. T., ed., *Lysosomes, a Laboratory Handbook*, Amsterdam: North-Holland Publishing Company, 46-135.

Bastian, E. D. and Brown, R. J. (1996) 'Plasmin in milk and dairy products: an update. *International Dairy Journal*, 6, 435-457.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemestry*, 72, 248-254.

Bockelmann, W., Hoppe-Seyler, T., Krush, U., Hoffmann, W. and Heller, K. J. (1997a) 'The microflora of Tilsit cheese. Part 2. Development of a surface smear starter culture', *Nahrung-Food*, 41, 213-218.

Bockelmann, W., Krush, U., Engel, G., Klijn, N., Smit, G. and Heller, K. J. (1997b) 'The microflora of Tilsit cheese. Part 1. Variability of the smear flora', *Nahrung-Food*, 41, 208-212.

Bockelmann, W., Hoppe-Seyler, T., Lick, S. and Heller, K. J. (1998) 'Analysis of casein degradation in Tilsit cheeses', *Kieler Milchwirtschafttliche, Forschungsberichte*, 50, 105-113.

Bosset, J. O. and Liardon, R. (1985) 'The aroma composition of Swiss Gruyère. III. Relative changes in the content of alkaline and neutral components during ripening', *Lebensmittel-Wissenchaft und-Technologie*, 18, 178-185.

Braun, S. D. and Olson, N. F. (1986) 'Microencapsulation of cell–free extracts to demonstrate the feasibility of heterogeneous enzyme systems and cofactor recycling for the development of flavour in cheese', *Journal of Dairy Science*, 69, 1202-1208.

Brew, K. (2003) 'α-Lactalbumin', *Advanced Dairy Chemistry, Volume 1: Proteins*, eds Fox, P. F. and McSweeney, P. L. H., 3rd edn, Part A, Kluwer Academic/Plenum Publishers, New York, 387-421.

Bruno, M. A., Pardo, M. F., Caffini, N. O. and López, L. M. I. (2002) 'Purification of a New Endopeptidase Isolated from Fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*)', *Acta Farmacéutica Bonaerense*, 21 (1), 51-56.

Carmona, M. A., Sanjuan, E., Gomez, R. and Fernández-Salguero, J. (1999) 'Effect of starter cultures on the physico-chemical and biochemical features in ewe cheese made with extracts from flowers of *Cynara cardunculus* L', *Journal of the Science of Food and Agriculture*, 79, 737-744.

Carretero, C., Trujillo, A. J., Mor-Mur, M., Pla, R. and Guamis, B. (1994) 'Electrophoretic study of casein breakdown during ripening of goat's milk cheese', *Journal of Agricultural and Food Chemistry*, 42, 1546-1550.

Casey, J. and Dobb, R. (1992) 'Microbial routes to aromatic aldehydes', *Enzyme and Microbial Technology*, 14, 739-747.

Cavalcanti, M. T. H., Teixeira, M. F. S., Lima Filho, J. L. and Porto A. L. F. (2004) 'Partial purification of new milk-clotting enzyme produced by *Nocardiopsis sp*', *Bioresource Technology*, 93, 29-35.

Charney, J. and Tomarelli, R. M. (1947) 'A colourimetric method for the determination of proteolytic activity of duodenal juice', *Journal of Biological Chemistry*, 171, 501-505.

Chazarra, S., Sidrach, L., Lopez-Molina, D. and Rodriguez-Lopez, J. (2007) 'Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus*, L.) flowers', *International Dairy Journal*, 17, 1393-1400.

Christen, G. L. and Marshall, R. T. (1984) 'Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media', *Journal of Dairy Science*, 67, 1680-1687.

Christensen, J. E., Dudley, E. G., Pederson, J. A. and Steele, J. L. (1999) 'Peptidases and amino acid catabolism in lactic acid bacteria', *Antonie Leeuwenhoek*, 76, 217-246.

Devaraj, K. B., Kumar, P. R. and Prakash, V. (2008) 'Purification, Characterization, and Solvent Induced Thermal Stabilization of Ficin from *Ficus carica*', *Journal of Agricultural and Food Chemistry*, 56, 11417-11423.

Doran, P. M. (2002) 'Properties and applications of hairy-root cultures', in Oksman-Caldenty, K.M. and Barz, W. H., eds., *Plant biotechnology and transgenic plants*, New York: Mercel Dekker Inc., 143-162.

Dunn, H. C. and Lindsay, R.C. (1985) 'Evaluation of the role of microbial Strecker-derived aroma compounds in unclean-type flavours of Cheddar cheese', *Journal of Dairy Science*, 68, 2859-2874.

Dziuba, J. and Minkiewicz, P. (1996) 'Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's κ-casein', *International Dairy Journal*, 6, 1017-1044.

Elsden, S. R., Hilton, M. G. and Waller, J. M. (1976) 'The end products of the metabolism of aromatic amino acids by *Clostridia*', *Archives of Microbiology*, 107, 283-288.

Englund, P. T., King, T. P., Craig, L. C. and Walti, A. (1968) 'Studies on ficin I. Its isolation and characterization', *Biochemistry*, 7, 163-175.

Faro, C. J., Moir, A. J. and Pires, E. V. (1992) 'Specificity of a milk clotting enzyme extracted from the thistle *Cynara cardunculus* L: Action on oxidized insulin and κ-casein. *Biotechnology Letters*, 14, 841-846.

Fernández-Salguero, J., Sanjuán, E. and Montero, E. (1991) 'A preliminary study of the chemical composition of Guía cheese', *Journal of Food Composition and Analysis*, 4, 262-269.

FernáNdez-Salguero, J. and Sanjuán, E. (1999) 'Influence of vegetable and animal rennet on proteolysis during ripening in ewes milk cheese', *Food Chemistry*, 64, 177-183.

Ferchichi, M., Hemme, D. and Boullianne, C. (1986a) 'Influence of oxygen and pH on methanethiol production from L-methionine by *Brevibacterium linens* CNRZ 918', *Applied and Environmental Microbiology*, 51, 725-729.

Ferchichi, M., Hemme, D. and Nardi, M. (1986b) 'Induction of methanethiol production by *Brevibacterium linens* CNRZ 918', *Journal of General Microbiology*, 132, 3075-3082.

Folkertsma, B., Fox, P. F. and McSweeney, P. L. H. (1996) 'Acceleration of Cheddar cheese ripeningat elevated temperatures', *International Dairy Journal*, 6, 1117-1134.

Fox, P. F. (1989) 'Proteolysis during cheese manufacture and ripening', *Journal of Dairy Science*, 72, 1379-1400.

Fox, P. F. (2003) 'Milk proteins: General and historical aspects', *Advanced Dairy Chemistry, Volume 1:Proteins*, eds. Fox , P. F. and McSweeney, P. L. H.), 3rd edn Part A, Kluwer Academic/Plenum, Publishers, New York, 1-48,

Fox, P. F., Singh, T. K. and McSweeney, P. L. H. (1995) 'Biogenesis of flavour compounds in cheese', in Malin, E. L. and Tunick, M. H., eds., *Chemistry of Structure/Function Relationships in Cheese*, London: Plenum Press, 59-98.

Fox, P. F. and McSweeney, P. L. H. (1996) 'Proteolysis in cheese during ripening', *Food Reviews International*, 12, 457-509.

Fox, P. F. and McSweeney, P. L. H. (1998) '*Dairy Chemistry and Biochemistry*', Blackie Academic & Professional, London.

Fox, P. F., McSweeney, P. L. H. and Lynch, C. M. (1998) 'Significance of non-starter lactic acid bacteria in Cheddar cheese', *Australian Journal of Dairy Technology*, 53, 83-89.

Fox, P. F. and Tobin, J. (1999) 'Acceleration and modification of proteolysis', *Proceedings of the 36th Annual Marshall Cheese Symposium*, Gateway to Success, Santa Clara, USA, September 9–10, 14-47.

Fox, P. F, Guinee, T. P, Cogan, T. M and McSweeney, P. L. H. (2000) 'Biochemistry of cheese ripening', in Springer, ed., *Fundamentals of Cheese Science*, Gaithersburg Maryland: Aspen Publishers, Inc., 236-281.

Fox, P. F. and Brodkorb, A. (2008) 'The casein micelle: historical aspects, current concepts and significance', *International Dairy Journal*, 18, 677-684.

Gao, S., Oh, D. H., Broadbent, J. R., Johnson, M. E., Weimer, B. C. and Steele, J. L. (1997) 'Aromatic amino acid catabolism by lactococci', *Lait*, 77, 371-381.

Green, M. L. (1972) 'Assessment of swine, bovine and chicken pepsin as rennet substitutes for cheddar cheese-making', *Journal of Dairy Research*, 39, 261-265.

Gripon, J. C. (1993) 'Mould-ripened cheeses', in Fox, P. F., ed., *Cheese: Chemistry, physics and microbiology, Vol. 2* , London: Chapman & Hall, 111-136.

Grufferty, M. B. and Fox, P. F. (1988) 'Milk alkaline proteinase [A review]', *Journal of Dairy Science*, 55, 609-630.

Guinee, T. M. and Wilkinson, M. G. (1992) 'Rennet coagulation and coagulants in cheese manufacture', *Journal of the Society of Dairy Technology*, 45, 94-104.

Hartley, B. S. (1960) 'Proteolytic enzymes', *Annual Review of Biochemistry*, 29, 45-72.

Heimgartner, U., Pietrzak, M., Geertsen, R., Brodelius, P., Silva-Figueiredo, A. C. and Pais M. S. S. (1990) 'Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*', *Phytochemistry*, 29, 1405-1410.

Hemme, D., Bouillane, C., Metro, F. and Desmazeaud, M. J. (1982) 'Microbial catabolism of amino acids during cheese ripening', *Sciences des Aliments 2*, 113-123.

Horne, D. S. and Banks, J. M. (2004) 'Rennet-induced coagulation of milk', *Chemistry, Physics and Microbiology, Volume 1: General Aspects*, eds Fox, P. F., McSweeney, P.L.H. Cogan, T.M. and Guinee, T.P., 3rd edn, Elsevier Academic Press, Amsterdam, 47-70.

Hummel, W. W. and Kula, M. R. (1984) 'Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity', *Archives of Microbiology*, 137, 47-52.

Hurley, W. L. (2003) 'Immunoglobulins in mammary secretions', *Advanced Dairy Chemistry, Volume 1: Proteins*, eds Fox, P.F. and McSweeney, P.L.H., 3rd edn, Part A, Kluwer Academic/Plenum Publishers, New York, 422-448.

International Union of Biochemistry (1992) 'Enzyme nomenclature', Academic Press, Inc., Orlando, Fla.

Kaminogawa, S. and Yamauchi, K. (1972) 'Acid protease of bovine milk. *Agricultural and Biological Chemistry*, 36, 2351-2356.

Karahadian, C. and Lindsay, R. C. (1987) 'Integrated roles of lactate, ammonia, and calcium in texture development of mold surface-ripening cheese', *Journal of Dairy Science*, 70, 909-918.

Kelly, A. L. and McSweeney, P. L. H. (2001) 'Indigenous proteinases', in: Fox, P. F. and McSweeney, P. L. H. , eds., *Advanced Dairy Chemistry-1. Proteins. (3rd ed.)*, Gaithersburg, MD: Aspen Publishers.

Kilcawley, K. N., Wilkinson, M. G. and Fox, P. F. (2002) 'Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources', *Enzyme and Microbial Technology*, 31, 310-320.

Kim, S. H., Choi, N. S., Lee, W. Y. (1998) 'Fibrin zymography: A direct analysis of fibrinolytic enzymes on gels, *Analytical Biochemistry*, 263, 115-116.

Kim, J. C. and Olson, N. F. (1989) 'Production of methanethiol in milk-fat coated microcapsules containing *Brevibacterium linens* and methionine', *Journal of Dairy Research*, 56, 799-811.

Kong, Y., Strickland, M. and Broadbent, J. R. (1996) 'Tyrosine and phenylalanine catabolism by L*actobacillus casei* flavour adjuncts: biochemistry and implications in cheese flavour', *Journal of Dairy Science*, 79, 101.

Kramer, D. E. and Whitaker, J. R. (1964) 'Ficus enzymes II. Properties of the proteolytic enzymes from the latex of *Ficus carica* variety kodata', *Journal of Biological Chemistry*, 239, 2178-2183.

de Kruif, C. G. and Holt, C. (2003) 'Casein micelle structure, functions, and interactions', *Advanced Dairy Chemistry, Volume 1: Proteins*, eds Fox, P. F. and McSweeney, P.L.H., 3rd edn, Part A, Kluwer Academic/Plenum Publishers, New York, 233-276.

Kunji, E. R. S., Mierau, I., Hagting, A., Poolman, B. and Konings, W. N. (1996) 'The proteolytic system of lactic acid bacteria', *Antonie van Leeuwenhoek*, 70, 187-221.

Jollivet, N., Bezenger, M. C., Vayssier, Y. and Belin, J. M. (1992) 'Production of volatile compounds in model milk and cheese media by eight strains of *Geotrichum candidum* Link', *Journal of Dairy Research*, 61, 241-248.

Joosten, H. M. L. J. and Stadhouders, J., (1987) 'Conditions allowing the formation of biogenic amines in cheese. 1: Decarboxylase properties of starter bacteria', *Netherland Milk and Dairy Journal*, 41 247-258.

Lane, C. N. and Fox, P. F. (1996) 'Contribution of starter and added lactobacilli to proteolysis in Cheddar cheese during ripening', *International Dairy Journal*, 6, 715-728.

Larsen, L. B., Benfeldt, C., Rasmussen, L. K. and Petersen, T. E. (1996) 'Bovine milk procathepsin D and cathepsin D: Coagulation and milk protein degradation', *Journal of Dairy Research*, 63, 119-130.

Law, B. A., Castanon, M. J. and Sharpe, M. E. (1976) 'Effect of non-starter bacteria on the chemical composition and flavour of Cheddar cheese', *Journal of Dairy Research*, 43, 117-125.

Law, J. and Haandrikman, A. (1997) 'Proteolytic enzymes of lactic acid bacteria', *International Dairy Journal*, 7, 1-11.

Leber, T. M. and Balkwill, F. R. (1997) ‘Zymography: A single-step staining method for quantitation of proteolytic activity on substrate gels’, *Analytical Biochemistry*, 249, 24-28.

Laemmli, U. K. (1970) ‘Cleavage of structural proteins during the assembly of the head of bacteriophage T4’, *Nature*, 227, 680-685.

Lemieux, L. and Simard, R. E. (1992) ‘Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition’, *Lait*, 72, 335-382.

Llorente, B. E., Brutti, C. B. and Caffini, N. O. (2004) ‘Purification and characterization of a milk-clotting aspartic proteinase from globe artichoke (*Cynara scolymus*, L.)’, *Journal of Agricultural and Food Chemistry*, 52, 8182-8198.

Lo Piero, A. R., Puglisi, I. and Petrone, G. (2002*)* ‘Characterization of ‘Lettucine,’’ a serine-like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting’, *Journal of Agriculture and Food Chemistry*, 50, 2439-2443.

Lynch, C. M., McSweeney, P. L. H., Fox, P. F., Cogan, T. M. and Drinan, F. D. (1997) ‘Contribution of starter lactococci and nonstarter lactobacilli to proteolysis in Cheddar cheese with controlled microflora’, *Lait*, 77, 441-459.

Macedo, A., Malcata, F. X. and Oliveira, J. C. (1993a) ‘The technology, chemistry, and microbiology of Serra cheese: A review’, *Journal of Dairy Science*, 76, 1725-1739.

Macedo, I. Q., Faro, C. J. and Pires, E. V. (1996) ‘Caseinolytic specificity of cardosin, an aspartic protease from the cardoon *Cynara cardunculus* L: Action on bovine α_s- and β-casein and comparison with chymosin’, *Journal of Agricultural and Food Chemistry*, 44, 42-47.

Madkor, S. A., Tong, P. S. and El Soda, M. (2000) ‘Ripening of Cheddar cheese with attenuated adjunct cultures of lactobacilli’, *Journal of Dairy Science*, 83, 1684-1691.

Manning, D. J. (1979a) ‘Chemical production of essential flavour compounds’, *Journal of Dairy Research*, 46, 531-537.

Manning, D. J. (1979b) ‘Cheddar cheese flavour studies. II: Relative flavour contributions of individual volatile components’, *Journal of Dairy Research*, 46, 523-529.

Marshall, J. D. and Cole W. M. (1983) ‘Threonine aldolase and alcohol dehydrogenase activities in *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* and their contribution to flavour production in fermented milks’, *Journal of Dairy Research*, 50, 375-379.

Martley, F. G. and Crow, V. L. (1993) 'Interactions between non-starter microorganisms during cheese manufacture and ripening', *International Dairy Journal*, 3, 461-483.

McCabe, B. J. (1986) 'Dietary tyramine and other pressor amines in MAOI regimens: a review', *Journal of the American Dietetic Association*, 86, 1059-1064.

McMahon, D. J. and Oommen, B. S. (2008) 'Supramolecular structure of the casein micelle', *Journal of Dairy Science*, 91, 1709-1721.

McSweeney, P. L. H., Fox, P. F. and Olson, N. F. (1995) 'Proteolysis of bovine caseins by cathepsin D: Preliminary observations and comparison with chymosin', *International Dairy Journal*, 5, 321-336.

McSweeney, P. L. H. and Sousa, M. J. (2000) 'Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review', *Lait*, 293-324.

Molimard, P. and Spinnler, H. E. (1996) 'Review: compounds involved in the flavour of surface moldripened cheeses: origins and properties', *Journal of Dairy Science*, 79, 169-184.

Mollé, D. and Leonil, J. (1995) 'Heterogeneity of the bovine κ-casein caseinomacropeptide, resolved by liquid chromatography on-line with electrospray ionization mass spectrometry', *Journal of Chromatography A*, 708, 223-230.

Mollé, D., Jean, K. and Guyomarch, F. (2006) 'Chymosin sensitivity of the heat-induced serum protein aggregates isolated from skim milk', *International Dairy Journal*, 16, 1435-1441.

Monnet, V., Chapot-Chartier, M. P. and Gripon, J.-C. (1993) 'Les peptidases des lactocoques', *Lait*, 73, 97-108.

Mora, M. T. and Marcos, A. (1981) 'Proteolysis del cheso Manchego: caseinas y productos de degradacion insolubles', *Archivos de Zootecnia*, 30, 1-17.

Muehlenkamp-Ulate, M. R. and Warthesen, J. J. (1999) 'Evaluation of several nonstarter lactobacilli for their influence on Cheddar cheese slurry', *Journal of Dairy Science*, 82, 1370-1378.

Muller, C. J., Kepner, R. E. and Webb, A. D. (1971) 'Identification of 3-(methylthio)-propanol as an aroma constituent in Cabernet Sauvignon and Ruby Cabernet wines', *American Journal of Enology and Viticulture*, 22, 953.

Mulvihill, D. M. and Fox, P. F. (1977) 'Proteolysis of α_{s1}-casein by chymosin: influence of pH and urea', *Journal of Dairy Research*, 44(3), 533-540.

Mulvihill, D.M. and Donovan, M. (1987) 'Whey proteins and their thermal denaturation – a review', *Irish Journal of Food Science and Technology*, 11, 43-75.

Nouani, A., Dako, E., Morsli, A., Belhamiche, N., Belbraouet, S. , Bellal, M.M. and Dadie, A. (2009) 'Characterization of the purified coagulant extracts derived from artichoke flowers (*Cynara scolymus*) and from the fig tree latex (*Ficus carica*) in light of their use in the manufacture of traditional cheeses in Algeria', *Journal of Food Technology*, 7(1), 20-29.

Nuňez, M., Fernández del Pozo, B., Rodriguez-Marin, M. A., Gaya, P. and Medina, M. (1991) 'Effect of vegetable and animal rennet on chemical, microbiological, rheological and sensory characteristics of La Serena Cheese', *Journal of Dairy Research*, 58, 511-519.

O'Connell, J. E. and Fox, P. F. (2000) 'The two-stage coagulation of milk proteins in the minimum of the heat coagulation time–pH profile of milk: effect of casein micelle size', *Journal of Dairy Science*, 83, 378-386.

Peterson, S. D. and Marshall, R. T. (1990) 'Nonstarter lactobacilli in Cheddar cheese: A review', *Journal of Dairy Science*, 73, 1395-1410.

Polo, C., Ramos, M., Sanchez, R. (1985) 'Free amino acids by high performance liquid chromatography and peptides by gel electrophoresis in Mahon cheese during ripening', *Food Chemistry*, 16, 85-96.

Ramalho-Santos, M., Veríssimo, P., Faro, C. and Pires, E. (1996) 'Action on bovine α_{s1}-casein of cardosins A and B, aspartic proteinases from the flowers of the cardoon *Cynara cardunculus* L.', *Biochimica et Biophysica Acta*, 1297, 83-89.

Raposo, S. and Domingos, A. (2008) 'Purification and characterization of milk-clotting aspartic proteinases from *Centaurea calcitrapa* cell suspension cultures', *Process Biochemistry*, 43, 139-144.

Raser, K. J., Posner, A. and Wang, K. K. W. (1995) 'Casein zymography: a method to study µ-calpain, m-calpain, and their inhibitory agents', *Archives of Biochemistry and Biophysics*, 319(1), 211-218.

Rattray, F. P., Fox, P. F. and Healy, A. (1996) 'Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine α_{s1}-casein', *Applied and Environmental Microbiology*, 62, 501-506.

Rattray, F. P. and Fox, P. F. (1997) 'Purification and characterization of an intracellular aminopeptidase from *Brevibacterium linens* ATCC 9174', *Lait*, 77, 169-180.

Rattray, F. P., Fox, P. F. and Healy, A. (1997) 'Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine β-casein', *Applied and Environmental Microbiology*, 63, 2468-2471.

Rattray, F. P. and Fox, P. F. (1999) 'Aspects of enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: A review', *Journal of Dairy Science*, 82, 891-909.

Roa, I., López, M. B. and Mendiola, F. J. (1999) 'Residual clotting activity and ripening properties of vegetable rennet from *Cynara cardunculus* in La Serena cheese', *Food Research International*, 32, 413-419.

Rollema, H. S., Mckellar, R. C., Sorhaugh, T., Suhren, G., Zadow, J. G., Law, B. A., Poll, J. K., Stepaniak, L. and Vagias, G. (1989) 'Comparison of different methods for the detection of bacterial proteolysis enzymes in milk', *Milchwissenschaft*, 44, 491-496.

Roseiro, L. (1991) 'Ewe's milk cheese making in Portugal using a vegetable rennet (a review)', *Sheep and Dairy News*, 8, 65-80.

Saito, T. and Ito, T. (1992) 'Variations and distributions of o-glycosidically linked sugar chains in bovine κ-casein', *Journal of Dairy Science*, 75, 1768-1774.

Santos, M. H. (1991) 'Biogenic amines: their importance in foods', *International Journal of Food Microbiology*, 29, 213-231.

Sawyer, L. (2003) 'β-Lactoglobulin', *Advanced Dairy Chemistry, Volume 1: Proteins*, eds Fox, P. F. and McSweeney, P. L. H., 3rd edn, Part A, Kluwer Academic/Plenum Publishers, New York, 319-386.

Sidrach, L., García-Cánovas, F., Tudela, J. and Rodríguez-López, J. N. (2005) 'Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A', *Phytochemistry*, 66, 41-49.

Sieber, R. and Lavanchy, P. (1990) 'Biogenic amines in dairy products and cheese', *Mitteilungen aus dem Gebiete der Lebensmittel-untersuchung un Hygiene*, 81, 82-105.

Sieber, R., Butikofer, U. and Bosset, J. O. (1995) 'Benzoic acid as a natural compound in cultured dairy products and cheese', International Dairy Journal, 5, 227-246.

Silva, S. V., Barros, R. M. and Malcata, F. X. (2002) 'Hydrolysis of caseins by extracts of *Cynara cardunculus* precipitated by ammonium sulphate', *Journal of Food Science*, 67, 1746-1751.

Smith, T. A. (1981) 'Amines in food', *Food Chemistry*, 6, 169-200.

Sousa, M. J. (1993) 'Plant rennet substitute from flowers of *Cynara cardunculus*', M.Sc., thesis, National University of Ireland, Cork, Ireland.

Sousa, M. J. C. F. (1998) 'Characterization of the proteolytic action of the flowers of Cynara cardunculus in ovine, caprine and bovine cheeses', Ph.D. Thesis, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal.

Sousa, M. J. and Malcata, F. X. (1996) 'Comparison of plant and animal rennets in terms of microbiological, chemical and proteolysis characteristics of ovine cheese', *Journal of Agricultural and Food Chemistry*, 45, 74-81.

Sousa, M. J. and Malcata, F. X. (1997a) 'Comparison of plant and animal rennets in terms of microbiological, chemical and proteolysis characteristics of ovine cheese', *Journal of Agricultural and Food Chemistry*, 45, 74-81.

Sousa, M. J. and Malcata, F. X. (1997b) 'Comparative biochemical evolution during ripening of bovine, ovine and caprine cheeses manufactured with extracts of flowers of *Cynara cardunculus*', *Zeitschrift f ũr Lebensmittel Untersuchung und-Forchung*, 205, 104-107.

Sousa, M. J. and Malcata, F. X. (1998a) 'Identification of peptides from ovine milk cheese manufactured with animal rennet or extracts of *Cynara cardunculus* as coagulant', *Journal of Agricultural and Food Chemistry*, 46, 4034-4041.

Sousa, M. J. and Malcata, F. X. (1998b) 'Proteolysis of ovine and caprine caseins in solution by enzymatic extracts from flowers of *Cynara cardunculus*', *Enzyme and Microbial Technology*, 22, 305-314.

Sousa, M. J., Ardö, Y. and McSweeney, P. L. H. (2001) 'Advances in the study of proteolysis during cheese ripening', *International Dairy Journal*, 11, 327-345.

Sumantha, A., Larroche, C. and Pandey, A. (2006) 'Food-grade proteases', *Food Technology and Biotechnology*, 44(2), 211-220.

Swaisgood, H. E. (2003) 'Chemistry of caseins', *Advanced Dairy Chemistry, Volume 1: Proteins*, eds Fox, P. F. and McSweeney, P. L. H.), 3rd edn, Part A, Kluwer Academic/Plenum Publishers, New York, 139-201.

Tamer, M. I. and Mavituna, F. (1997) 'Protease from freely suspended and immobilized *Mirabilis jalapa*', *Process Biochemistry*, 32, 195-200.

Tamine, A. Y. and Deeth, H. C. (1980) 'Yogurt, technology and biochemistry', *Journal of Food Protection*, 43, 939.

Tan, P. S. T., Poolman, B. and Konings, W. N. (1993) 'Proteolytic enzymes of *Lactococcus lactis*', *Journal of Dairy Research*, 60, 269-286.

Tavaria, F. K., Sousa, M. J., Domingos, A., Malcata, F. X., Brodelius, A., Clemente, A. and Pais, M. S. (1997) 'Degradation of caseins from different milk types by extracts of *Centaurea calcitrapa*', *Journal of Agricultural and Food Chemistry*, 45, 3760-3765.

Tavaria, F. K., Sousa, M. J. and Malcata, F. X. (2001) 'Storage and lyophilization effects of extracts of *Cynara cardunculus* on the degradation of ovine and caprine caseins', *Food Chemistry*, 72, 79-88.

Taylor, S. L., Keefe, T. J., Windham, E. S. and Howell, J. F. (1982) 'Outbreak of histamine poisoning associated with consumption of Swiss cheese', *Journal of Food Protection*, 45, 455-457.

Thomas, T. D. and Pritchard, G. C. (1987) 'Proteolytic enzymes in dairy starter cultures', *Federation of European Microbiological Assocciations Microbiology Review*, 46, 245-268.

Urbach, G. (1995) 'Contribution of lactic acid bacteria to flavour compound formation in dairy products', *International Dairy Journal*, 5, 877-903.

Veríssimo, P., Esteves, C., Faro, C. and Pires, E. (1995) 'The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities', *Biotechnology Letters*, 17, 621-626.

Veríssimo, P., Faro, C. J., Moir, A. J., Lin, Y., Tang, J. and Pires, E. (1996) 'Purification, characterization and partial amino acid sequencing of two new aspartic proteinases from fresh flowers of *Cynara cardunculus* L.', *European Journal of Biochemistry*, 235, 762-768.

Vioque, M., Gómez, R., Sánchez, E., Mata, C., Tejada, L. andFernández-Salguero, J. (2000) 'Chemical and microbiological characteristics of ewes milk cheese manufactured with extracts from flowers of *Cynara cardunculus* and *Cynara humilis*', *Journal of Agriculture and Food Chemistry*, 48, 451-456.

Visser, S. (1993) 'Proteolytic enzymes and their relation to cheese ripening and flavour [An Overview]', *Journal of Dairy Science*, 76, 339-350.

Voight, M. N. and Eitenmiller, R. R. (1978) 'Role of histidine and tyrosine decarboxylases and mono–and diamine oxidases in amine building–up in cheese', *Journal of Food Protection*, 41, 182-186.

Williams, A. G. and Banks, J. M. (1997) 'Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria isolated from Cheddar cheese manufactured in the United Kingdom', *International Dairy Journal*, 7, 763-774.

Williams, A. G., Xavier, F. and Banks, J. M. (1998) 'Aminopeptidase and dipeptidyl peptidase activity of *Lactobacillus* spp. and nonstarter lactic acid bacteria (NSLAB) isolated from Cheddar cheese', *International Dairy Journal*, 8, 255-266.

Yadav, S. C., Pande, M. and Jagannadham, M. V. (2006) 'Highly stable glycosylated serine protease from the medicinal plant *Euphorbia milii*', *Phytochemistry*, 67(14), 1414-1426.

Yvon, M., Thirouin, S., Rijnen, L., Fromentier, D. and Gripon, J. C. (1997) 'An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavour compounds', *Applied and Environmental Microbiology*, 63, 414-419.

Zoon, P. and Allersma, D. (1996) 'Eye and crack formation in cheese by carbon dioxide from decarboxylation of glutamic acid', *Netherland Milk and Dairy Journal*, 50, 309-318.

CHAPTER 4

Antioxidant activity of hydrolysates of bovine casein

ABSTRACT

The antioxidant activity of bioactive peptides can be attributed to their radical scavenging, inhibition of lipid peroxidation and metal ion chelation properties. *Cynara cardunculus* L. and *Ficus carica* L. proteinases were investigated for their ability to produce antioxidant hydrolysates/peptides from bovine casein (CN). Since *Cynara cardunculus* protease reported low degree of hydrolysis (DH) on Na-CN, this plant extract was not considered as a good source of bioactive peptides. On the contrary, Na-CN and β-CN hydrolysates obtained with *Ficus carica* protease had a final degree of hydrolysis (DH) of 18.49 and 21.24%, respectively, after 6 and 4 h digestion. Consistent with the changes in DH, the hydroxyl radical scavenging activity increased with increasing DH. The Oxigen Radical Absorbance Capacity (ORAC) values of Na-CN and β-CN hydrolysates varied and ranged from 6506 to 18081, and from 50722 to 118977 μmol Trolox/100g of protein, respectively. Gel permeation HPLC showed that the 21.24 % DH β-CN hydrolysate had 64.88 % of peptide material with MW < 500 Da. Analysis of β-CN hydrolysate by UPLC/MS allowed identification of 12 peptide sequences with potential antioxidant properties due to their high level of His, Leu, Pro residues and other hydrophobic amino acids. The results suggested that the antioxidant activity of bovine casein hydrolysates and, especially β-CN digests, were related to their degree of hydrolysis (DH) as well as to the amino acid composition and sequence, the size of peptides produced at different stages of digestion.

Keywords: *Cynara cardunculus* L., *Ficus carica* L., sodium caseinate, β-casein, degree of hydrolysis, bioactive peptides, antioxidant activity, $ORAC_{FL}$ assay, mass spectrometry.

INTRODUCTION

The role of proteins in the diet is widely recognized, not only as a source of essential amino acids but also of biologically active peptides (BAPs). Bioactive peptides have been

defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003) by affecting the cardiovascular, digestive, immune and nervous systems. These peptides are not active within the parent protein but can be released and activated following enzymatic hydrolysis (FitzGerald and Meisel, 2003; Gobbetti *et al.*, 2002). The activity of these peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to contain multifunctional properties (Meisel and FitzGerald, 2003). In this regard, milk proteins are considered the most important source of bioactive peptides as reviewed in many articles (FitzGerald and Meisel, 2003; Korhonen and Pihlanto, 2003a, b; Silva and Malcata, 2004; Korhonen and Pihlanto, 2006; Hartmann and Meisel, 2007; Korhonen, 2009; Phelan *et al.*, 2009a).

This chapter focuses on casein-derived peptides, with particular emphasis on antioxidant peptides for three main reasons. At first, the identification of antioxidative BAPs, which could interact with free radicals and other reactive oxygen species (ROS) by preventing oxidative damage in the body and in foodstuffs, is highly desirable. Therefore, research focusing on this area is beneficial. Secondly, recent studies have shown that antioxidative peptides can be released from caseins during hydrolysis by digestive enzymes and during fermentation of milk with proteolytic LAB strains (Korhonen and Pihlanto, 2003b). At present, most of antioxidative casein derived-peptides have been generated by enzymatic hydrolysis with pepsin and trypsin (Suetsuna *et al.*, 2000; Rival *et al.*, 2001b), and fermentation of milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* (Kudoh *et al.*, 2001). However, there is a lack of studies on the use of plant proteinases and/or coagulants in the release of antioxidant peptides from casein. Hence, further investigations are needed to enhance the availability of bioactive peptides and, in particular, to promote the production of antioxidants using natural sources, such as plant proteinases. Finally, the commercial use of proteinases of plant-origin has gained increased popularity due to their unique functions and cost-effectiveness (Hogan *et al.*, 2009). In this regard, the use of plant-derived proteinases to generate plant-derived bioactive peptides can meet the needs of people who avoid consuming animal-derived products due to diet (vegetarians) and/or religious reasons (e.g., Judaism and Islamism).

Ficus carica L. and *Cynara cardunculus* L. are mainly known to produce proteinases which coagulate milk. Several studies have purified and characterized proteinases from the latex of *Ficus carica* L. (Sgarbieri *et al.*, 1964; Devaraj *et al.*, 2008; Azarkan *et al.*, 2001) and the aqueous extract of *Cynara cardunculus* L. (Lo Piero *et al.*, 2002; Bruno *et al.*, 2002; Sidrach

et al., 2004). However, to our knowledge no previous studies appear to report on the use *Ficus carica* and *Cynara cardunculus*- derived proteinases in the release of antioxidative BAPs. The objective of the current study was to evaluate the antioxidant activity of bovine sodium caseinate (Na-CN) and β-casein (β-CN) hydrolysates generated using these two plant extracts.

1. GENERATION OF BIOACTIVE PEPTIDES

Biologically active peptides can be produced from precursor milk proteins in the following ways: (a) enzymatic hydrolysis by digestive enzymes, (b) fermentation of milk with proteolytic starter cultures, and/or (c) proteolysis by enzymes derived from microorganisms or plants. In many studies combinations of (a), (b) and (c) have proven effective in the generation of biofunctional peptides (Korhonen and Pihlanto, 2003a).

1.1 ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis of whole proteins is the main approach used to produce bioactive peptides. Digestive enzymes, such as pepsin, trypsin and chymotrypsin, have been shown to release bioactive peptides from various proteins. In addition, enzyme combinations including Alcalase^{TM}, chymotrypsin, Pancreatin, trypsin and thermolysin as well as enzymes from bacterial and fungal sources can be used to generate various bioactive peptides from different intact protein molecules (FitzGerald *et al.*, 2004; Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003b).

1.2 MICROBIAL FERMENTATION

Many dairy starter cultures are highly proteolytic. Lactic acid bacteria (LAB), such as *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* var. *bulgaricus*, can generate bioactive peptides when used in the manufacture of fermented dairy products. Many authors have reported the release by microbial fermentation of various bioactive peptides from milk (Nakamura *et al.*, 1995; Pihlanto-Leppälä *et al.*, 1998; Yamamoto *et al.*, 1999).

1.3 HYDROLYSIS USING ENZYMES OBTAINED FROM MICROORGANISMS

Proteolytic enzymes isolated from LAB have also been successfully used to release bioactive peptides from milk proteins. For example, Yamamoto *et al.* (1994) studied the

angiotensin-I-converting-enzyme (ACE) inhibitory activity of peptides derived from α_{s1}- and β-casein by employing a proteinase from *Lactobacillus helveticus* CP790. Other authors purified and characterised an endopeptidase from *Lactobacillus helveticus* CM4 and demonstrated that this peptidase can generate anti-hypertensive peptides using synthetic pro-peptides as substrates (Ueno *et al.*, 2004). The use of commercially available microbially-derived food grade proteinases to hydrolyse casein is advantageous as these enzymes are low-cost and safe (Mao *et al.*, 2007).

2. BIOLOGICAL ACTIVITIES OF CASEIN HYDROLYSATES

A growing interest has focused on biologically active casein-derived peptides as regulators of specific physiological functions in the body. These peptides are inactive within the sequence of the parent protein molecule and can be liberated by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing (Kitts and Wailer, 2003). The beneficial health effects may be attributed to numerous known peptide sequences exhibiting antimicrobial, antioxidative, antithrombotic, antihypertensive, immunomodulatory and opioid activities (Korhonen, 2009). Moreover, some peptides are multifunctional and can exert more than one of these activities (Meisel, 2004).

2.1 HYPOTHENSIVE (ACE INHIBITORY) PEPTIDES

Angiontensin-I-converting enzyme (ACE), a zinc-dependent carboxypeptidase, has been associated with the rennin-angiotensin system which regulates peripheral blood pressure. The enzyme catalyzes the conversation of angiotensin I to the potent vasoconstrictor angiotensin II with a consequent increasing of blood pressure. ACE can also catalyze the degradation of bradykinin, a vasodilatory peptide, and enkephalins. It is evident, therefore, that inhibition of ACE may exert an anthihypertensive effect as a consequence of a decrease in angiontensin II and a concomitant increase in bradykinin activity (FitzGerald and Meisel, 2003). A great number of ACE inhibitory peptides have been identified from the digestion of milk proteins and in particular from caseins. Casokinin sequences, casein-derived peptide inhibitors of ACE (Meisel and Schlimme, 1994), have been found in α_{s1} , β- and κ-caseins. The IC_{50} value (inhibitor concentration leading to 50% inhibition) is used to estimate the effectiveness of different ACE inhibitory peptides (Hartmann and Meisel, 2007). For example, potent casokinins include α_{s1}-CN (f 25-27), β-CN (f 74-76), β-CN (f 169-174), κ-CN (f 108-110) which have ACE IC_{50} values of 2, 5, 5 and 5 μmol/l, respectively (FitzGerald and Meisel, 2003).

2.2 OPIOID PEPTIDES

Opioid peptides are defined as peptides such as enkephalins, which have an affinity for an opiate receptor as well as opiate-like effects. These opioid peptides are opioid receptors ligands with agonistic or antagonistic activities. Opioid receptors are located in the nervous, endocrine and immune systems as well as in the gastrointestinal tract of mammals. The physiological effect of these peptides depends on receptor type, e.g., μ receptors are linked to the control of intestinal motility and emotional behaviour, δ receptors control emotional behaviour and κ receptors are linked with analgesia and satiety (Höllt, 1983). The common structural motif of opioid peptides is an N-terminal tyrosine residue (except α-casein opioids) and another aromatic residue in the third or fourth position from the N terminus (Phe or Tyr) (Meisel, 1998).

Peptides with opioid activity have been identified in various casein fractions hydrolyzed by digestive enzymes (Loukas *et al.*, 1983; Xu, 1998; Perpetuo *et al.*, 2003). The first exogenous opioid peptides discovered were β-casomorphins (Brantl *et al.*, 1979), which are fragments of β-casein between (f 60-70) and were characterized as μ-type ligands (Meisel, 1986; Teschemacher and Brantl, 1994). Three α_s-casein-derived exorphins, which correspond to bovine α_{s1}-casein f 90–95, f 90–96 and f 91–96, were found to be δ-selective receptor ligands (Loukas et al, 1983). More rencently, Perpetuo *et al.* (2003) reported a new peptide from γ-casein, Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu, possessing *in vitro* opiate-like activity, which was generated by tryptic hydrolysis of casein. Opioid antagonists have been found in bovine κ-casein (Chiba et al, 1989). Two bovine casoxins are opioid receptor ligands of the μ-type, with relatively low antagonistic potency as compared with naloxone (Meisel, 1998). Casoxins A and B correspond to amino-acid sequences within bovine κ-casein; casoxin A corresponds to f 35–41 of κ-casein (i.e. Tyr-Pro-Ser-Tyr-Gly-Leu-Asn), whereas casoxin B is accounted for by f 58–61 of κ-casein (i.e. Tyr-Pro-Tyr-Tyr). Casoxin C is an opioid antagonist obtained from tryptic digests of bovine κ-casein; its sequence corresponds to f 25–34 (i.e. Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg), and possesses the highest biological potency (Xu, 1998).

2.3 MINERAL BINDING PEPTIDES (CCPS)

Tryptic digestion of the casein proteins yields caseinophosphopeptides (CCPs), which contains clusters of phosphorylated seryl residues (Cross *et al.*, 2005). These phosphoseryl clusters have been hypothesized to be responsible for the interaction between the caseins and calcium phosphate that leads to the formation of casein micelles. The excellent bioavailability of minerals such as calcium from dairy products has been attributed, in part to the presence of

caseinophophopeptides (CPPs) which, due to their highly polar acidic domains, can binds and solubilise minerals (Kitts and Yuan, 1992; FitzGerald, 1998). CPPs have been found after *in vitro* and/or *in vivo* digestion of α_{s1} -, α_{s2} and β-casein (Kitts, 1994). However, until now, no evidence has been supplied for the effectiveness of CPPs in increasing passive calcium absorption in humans (Meisel, 2007).

2.4 ANTIMICROBIAL PEPTIDES

The most well-studied antimicrobial peptides from milk are the lactoferricins, which derive from the whey protein, lactoferrin. However, a few antibacterial peptides have been identified from α_{s1} and α_{s2}- casein (Rizzello *et al.*, 2005; McCann *et al.*, 2006). In particular, an antibacterial isracidin, α_{s1}-CN (f 1-23), was significantly effective against lethal infection of mice by *Staphylococcus aureus*, but also *Candida albicans*. Furthermore, caseicidin, obtained by chymosin-mediated digestion of casein at neutral pH, was among the first defence peptides actually purified - it exhibited activity against *Staphylococcus* spp., *Sarcina* spp., *Bacillus subtilis*, *Diplococcus pneumoniae* and *Streptococcus pyogenes* (Lahov and Regelson, 1996). Generally, antimicrobial peptides act against different Gram-positive and Gram-negative bacteria (*Escherichia*, *Helicobacter*, *Listeria*, *Salmonella* and *Staphylococcus*), yeasts and filamentous fungi (Meisel, 2007).

2.5 IMMUNOMODULATORY PEPTIDES

Immunomodulatory peptides can enhance immune cell functions, measured as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis and cytokine regulation (Horiguchi *et al.*, 2005; FitzGerald and Murray, 2006). Moreover, immunomodulatory peptides may reduce allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract (Korhonen and Pihlanto, 2003). In particular, peptides obtained during hydrolysis of casein have been shown to modulate various aspects of immune function (Gill *et al.*, 2000). Immunomodulatory peptides derived from bovine β-casein (f 63–68 and f 191–193) and α_{s1}-casein (f 194–199) were isolated (Migliore-Samour and Jollès, 1988). These peptides were found to stimulate phagocytic activity of murine and human macrophages *in vitro*, and exert a protective effect against *Klebsiella pneumoniae* infection in mice *in vivo* (Migliore-Samour, Floch and Jollès, 1989). More recently, the pH 4.6-soluble products from the hydrolysis of whole bovine casein by chymosin encompassed peptides possessing immunomodulatory activity, i.e. α_{s1}-casein f 1–23 and β-casein f 193–209 (Minkiewicz *et al.*, 2000). A tripeptide from κ-casein significantly increased the proliferation

of human peripheral blood lymphocytes *in vivo* (Kayser and Meisel, 1996). The C-terminal sequence of bovine β-casein (f 193–209), containing β-casokinin-10, induced similar results in rats (Coste *et al.*, 1992)

2.6 Cytomodulatory Peptides

Cytochemical studies have provided increasing evidence that food-derived bioactive peptides modulate viability (e.g. proliferation, differentiation and apoptosis) of different cell types. Some milk-derived peptides, for example, have been shown to trigger apoptosis, especially in malignant cells, whereas normal cells seem to be less susceptible. Cytomodulatory peptides derived from casein fractions inhibit cancer cell growth or stimulate the activity of immunocompetent cells and neonatal intestinal cells (Meisel and FitzGerald, 2003).

2.7 Antithrombotic Peptides

Antithrombotic peptides, casoplatelins, are derived from the C-terminal region (caseinoglycomacropeptide) of bovine κ-casein. These peptides inhibit blood platelet aggregation and fibrinogen binding (γ-chain). The main antithrombotic peptides of κ-casein correspond to κ-CN (f 106-116). The smaller peptides, (f 106-112) and (f 113-116), are less potent inhibitors of platelet aggregation and do not inhibit fibrinogen binding (Fiat and Jollès, 1989; FitzGerald and Meisel, 2003).

2.8 Antioxidant Peptides

2.8.1 Oxidative Damage

Free radicals and other reactive oxygen species (ROS) contribute to oxidation processes in the body and in foodstuffs. Oxidative damage causes several age-specific diseases and neurodegenerative disorders. For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients (Halliwell, 2001; Butterfield, 2002; Liu *et al.*, 2003). Other diseases in which oxidative damage has been implicated include cancer, atherosclerosis, other neurodegenerative diseases and diabetes (Hagen *et al.*, 1994; Chowienczyk *et al.*, 2000; Halliwell, 2000; 2001; 2002a, b; Parthasarathy *et al.*, 2000). Compounds which are responsible for destructive and lethal cellular effects are lipid peroxides and low molecular weight compounds produced during the late stage of oxidative reactions. The main targets of these reactions are lipids, proteins, DNA and enzymes (Halliwell *et al.*, 1995). It is also well

known that oxidative reactions in food also lead to deterioration in quality, such as unacceptable taste or texture, colour, loss of nutritive value and shortening of shelf life. Therefore, it is important to inhibit lipid peroxidation to prevent the loss of quality in foodstuffs and to protect the body against age-specific diseases and neurodegenerative disorders. The use of synthetic and natural antioxidants can prevent and diminish oxidative stress and its deleterious effects. Synthetic antioxidants are cost-effective and efficient but display some toxic and hazardous effects (Ito *et al.*, 1985), consequently their use in foodstuffs is restricted or prohibited in some countries. On the contrary, natural antioxidants have been the focus of growing interest for their potential health benefits with no or little side effects (Sarmadi and Ismail, 2010).

2.8.2 ANTIOXIDATIVE PEPTIDES FROM CASEIN

Proteins from certain food sources have been reported to have the ability to scavenge active oxygen species (Okada and Okada, 1998). Food proteins displaying antioxidant activity, include porcine myofibrillar protein (Saiga *et al.*, 2003), soy and gelatin (Park *et al.*, 2005), potato (Pihlanto *et al.*, 2008), milk proteins of which caseins (Rival *et al.*, 2001a, b) and finally whey proteins (Hu *et al.*, 2003a,b; Lajoie *et al.*, 2011; Peña-Ramos and Xiong, 2003). In addition, protein hydrolysates have also been found to exhibit antioxidant activity. In this regard, the antioxidant potential of proteins derived from dairy products is well known (Allen and Wrieden, 1982; Colbert and Decher, 1991; Stuchell and Krochta, 1995; Maté *et al.*, 1996). Caseins have polar domains that contain phosphoseryl residues, and their characteristic sequences, –SerP– SerP–SerP–Glu–Glu, are effective cation chelators that form complexes with calcium, iron and zinc. Thus, phosphorylated caseins and/or their peptides in the aqueous phase could be a source of natural chelators to control lipid oxidation in food emulsions by binding and partitioning transition metals away from the emulsion droplet (Pihlanto, 2006). Cervato *et al.* (1999) have studied the antioxidant properties of milk casein subunits in liposomal models. All the subunits of casein inhibited Fe induced peroxidation of arachidonic derived liposomes. Antioxidant properties might, therefore not be uniquely attributed to whole caseins but also peptides generated from their digestion. Rival *et al.* (2001b) evaluated the effects of various types of bovine casein on the activity of soybean lipoxygenase *in vitro* and identified the domain within the protein responsible for the inhibitory activity. They found that a tryptic β-casein digest and clostripain and subtilisin digests of whole casein retained their inhibitory properties. Moreover, the highest inhibition of linoleic acid oxidation was observed in the fraction containing β-casein f (169–176) with a

trace amount of β-casein f (33–48). In another study, Rival *et al.* (2001a) assessed the structure-function relationship between the amino acid sequence, antioxidant capacity and effectiveness by using three different methods. They observed that casein-derived peptides inhibited enzymatic and non-enzymatic lipid peroxidation and concluded that the antioxidative feature was not lost with the dephosphorylation or on proteolysis of the proteins. Other authors (Kansci *et al.*, 2004) reported that the β-casein tryptic peptide (f 1–25) may protect polyunsaturated fatty acid (PUFA)-rich phospholipid liposomes against long-term iron-induced oxidation, mainly due to iron chelation. Diaz *et al.* (2003) reported that casein hydrolysates were more effective inhibitors of lipid oxidation in oil-in-water emulsions than enriched CPPs at equal phosphorus content. Diaz and Decker (2004) compared the antioxidant activity of CPPs and casein hydrolysates in a cell membrane model system of phosphatidylcholine liposomes and in ground beef. They reported that iron-induced lipid oxidation was inhibited by both casein hydrolysates and CPPs, with casein hydrolysates being less effective than CPPs. Research on various casein hydrolysates has also shown that they also possess radical scavenging activities. Suetsuna *et al.* (2000) isolated and identified free radical scavenging activity from a peptic digest of casein. The peptide, Tyr-Phe-Tyr-Pro-Glu-Leu from α_{s1}-casein f(144–149), was found to possess a potent superoxide anion radical scavenging activity and it was showed that the C-terminal dipeptide Glu-Leu sequence played an important role in this activity. Furthermore, this peptide compared with the standard substances such as glutathione and carnosine, showed higher scavenging activity for hydroxyl radicals. Generally, antioxidant peptides have been identified from the major casein fractions, i.e., β-casein (Rival *et al.*, 2001a, b;) and α_{s1}-casein (Suetsuna *et al.*, 2000; Srinivas and Prakash, 2010). However, a limited number of studies (Kudoh *et al.*, 2001; López.Expósito *et al.*, 2007) have reported on the identification of antioxidant peptides from the minor caseins (κ or α_{s2}-casein).

2.8.3 HEALTH EFFECTS

A limited number of researchers report on the health effects of antioxidative peptides from milk. The results of some animal and human studies suggest that fermented milk products have an antioxidative effect. A fermented milk product was found to exert an antiperoxidative action on rats fed a vitamin-E deficient diet. The lactic acid bacteria and whey proteins, particularly β-lactoglobulin, in the product contributed partly to the observed antiperoxidative action (Zommara *et al.*, 1998). Kullisaar *et al.* (2003) noted that the consumption of fermented goat milk improved anti-atherogenicity in healthy subjects. This

was thought to be via prolonging the resistance of the lipoprotein fraction to oxidation, lowering the levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and the glutathione redox ratio, and enhancing total antioxidative activity (Pihlanto, 2006). More studies are needed to enhance current knowledge of the role of antioxidative peptides in the protection of humans and to demonstrate their potential health benefits.

3. MATERIALS AND METHODS

3.1 STANDARD AND REAGENTS

Sodium caseinate (Na-CN) was obtained from the Kerry Ingredients (Listowel, Co. Kerry, Ireland). Sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), Bradford reagent, β-casein, sodium lauryl sulphate (SDS), Trolox, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein disodium, trifluoroacetic acid (TFA), L-tyrosine, L-leucine, β-mercaptoethanol, glycine, trizma base, glycerol, ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), low-molecular weight marker proteins, N,N'-methylene bis-acrylamide, and acrylamide (all electrophoretic grade) were supplied by Sigma Aldrich (Dublin, Ireland). 2,4,6-trinitrobenezene sulfonic acid (TNBS) was obtained from Medical Supply Co (Damastown, Co. Dublin, Ireland). HPLC grade acetonitrile, HPLC grade water and PTFE syringe filters (0.2 µm) were obtained from VWR (Dublin, Ireland). A microplate fluoroscence reader (Biotek Synergy HT) was used with fluorescence filters having an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The plate reader was controlled by Gen 5 software (Biotek). Black 96-well microplates were purchased from Thermo Fisher Scientific (Dublin, Ireland).

3.2 PLANT MATERIALS

To obtain a representative sample, latex was collected from *Ficus carica* L. trees, under the same environmental and soil characteristics, in the Puglia region (Foggia, Italy). All samples were harvested manually at the same stage of maturation (August 2011), by incising the stalk of the green fruit from the main branch. Several drops of the latex from the fig were allowed to drip into the test tubes. It was clarified by centrifugation at 14000g at 4°C for 15 min. The clear supernatant (serum) was separated from the gummy material and stored at –20°C until further use.

3.3 Protein Determination

Protein concentration was determined by the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein.

3.4 Hydrolysis of Sodium Caseinate

Sodium caseinate (Na-CN), at protein concentrations of bovine milk 3.5% (w/v), was hydrolyzed, respectively, with the *Cynara cardunculus* L. crude extract and *Ficus carica* L. serum at 37°C in a thermostated reaction vessel. The enzyme-to-substrate ratios (E:S) used were calculated on the basis of protein content in the enzyme and the caseinate solutions. At defined time intervals, 250 mL of Na-CN hydrolysate were removed and the enzyme was inactivated by heating at 90°C for 20 min. Samples stored at -20°C were freeze-dryed for subsequent analysis.

3.5 Hydrolysis of β-casein

β-casein (β-CN), at protein concentrations of bovine milk 1% (w/v), was hydrolyzed with the *Ficus carica* L. serum at 37°C in a thermostated reaction vessel. The enzyme-to-substrate ratio (E:S) used, (1:10), was calculated on the basis of protein content in the enzyme and the caseinate solutions. At defined time intervals, 25 mL of β-CN hydrolysate was removed and the enzyme was inactivated by heating at 90°C for 20 min. Samples stored at -20°C were freeze-dryed for subsequent analysis.

3.6 Quantification of Degree of Hydrolysis (DH) Using TNBS

Degree of hydrolysis (DH%), defined as the percentage of peptide bonds hydrolyzed, was determined using the trinitro-benzene-sulfonic acid (TNBS) method of Adler-Nissen (1979a). The TNBS reagent consisted of 0.1% (w/v) TNBS in methanol. All samples and standard solutions were prepared in 1% (w/v) SDS. Triplicate aliquots (0.125 mL) of test or standard solutions were added to test tubes containing 1.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2). TNBS reagent (1.0 mL) was then added to each tube, followed by mixing and incubation at 50°C for 60 min in a covered water bath (to exclude light). After incubation, the reaction was stopped by the addition of 0.1 M HCl (2.0 mL) to each tube. Samples were then allowed to cool at room temperature for 30 min, before absorbance values were measured at 340 nm using a spectrophotometer. L-Leucine (0- 2.0 mM) was used to generate a standard curve. DH values were calculated using the following formula (Adler-Nissen, 1979b):

$$DH\% = 100\left(\frac{AN_2 - AN_1}{Npb}\right)$$

where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg g^{-1} protein), AN_2 the amino nitrogen content of the protein substrate after hydrolysis (mg g^{-1} protein), and Npb the nitrogen content of the peptide bonds in the protein substrate (mg g^{-1} protein). A value of 112.1 was used for casein protein (Adler-Nissen, 1979b). The values of AN_1 and AN_2 were obtained by reference to a standard curve of Abs at 340 nm versus mg L^{-1} amino nitrogen (generated with L-Leucine). These values were then divided by the protein content of the test samples to give mg amino nitrogen per gram of protein.

3.7 Electrophoretic Characterization of Hydrolysates

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to examine the time-dependent proteolysis of NaCN by *Cynara cardunculus* protease extract. SDS-PAGE was performed in a vertical gel apparatus Mini-Protean II (Bio-Rad Laboratories, Milan, Italy) on 16% (w/v) according to the procedure of Laemmli (1970). Hydrolysate samples containing approximately 10 µg of protein were treated with loading buffer (0.5 M Tris-HCl pH 6.8, 20% glycerol, 4.4% SDS, 10 mg/mL bromophenol blue and 300 mM β-mercaptoethanol) and heated at 100 °C for 5 min then boiled for 5 min before SDS-PAGE. Electrophoresis was run at 100 V until the tracking dye bromophenol blue disappeared from the separating gel. After electrophoresis, the gels were stained with 2 g Coomassie brilliant blue R-250 (in a 10:40:50 solution of acetic acid: methanol: water) and de-stained with a 10:40:50 solution of acetic acid: methanol: water. The molecular mass standards (Bio-Rad Laboratories, Milan, Italy) were bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), bovine carbonic anhydrase (29.0 kDa), bovine trypsinogen (24.0 kDa), trypsin inhibitor (20.0 kDa); bovine α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

3.8 Characterization of Antioxidant Activity ($ORAC_{FL}$)

The antioxidant activity was determined by using the $ORAC_{FL}$ assay according to the method of Zulueta *et al.* (2009) with some modifications. Briefly, the reaction was performed at 37°C in 75 mM phosphate buffer (pH 7.0) and the final assay mixture (125 µL) contained fluorescein (FL, 0.78 µM), 2,2´ -azobis(2-amidinopropane) dihydrochloride (AAPH) radical

(221 mM), the standard Trolox (0- 120 µM) or sample at different concentrations. The plate was automatically shaken before the first reading and the fluorescence was recorded every 5 min for 120 min, until the relative fluorescence intensity (RFI%) was less than 5% of the value of the initial reading. A microplate reader with fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 530 nm was used. Black 96-well microplates were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM PBS (pH 7.0). The ORAC assay was performed with three independent trials (replications) and the measurements were taken in triplicate. $ORAC_{FL}$ values were determined by using the area under the fluorescein decay curve (AUC):

$$\text{AUC} = \sum \frac{(FI_1)}{FI_0} \ldots. \frac{(FI_n)}{FI_0} \cdot 5$$

where FI_0 is the initial fluorescence and FI_n is the fluorescence at time n. The net AUC corresponding to a sample was calculated as follows:

$$\text{Net AUC} = \text{AUC sample-AUC blank}$$

The final $ORAC_{FL}$ values were expressed as µmol of Trolox equivalents per 100 grams of solid sample.

3.9 GEL PERMEATION HIGH-PERFORMANCE LIQUID CROMATOGRAPHY (GP-HPLC)

Molecular mass distribution profiles of hydrolysed samples were obtained by gel permeation high performance liquid chromatography (GP-HPLC) using a Waters HPLC system, comprising a model 1525 binary pump, a model 717 Plus autosampler and a model 2487 dual λ absorbance detector interfaced with a Breeze data-handling package (Milford, MA). Hydrolysate samples were prepared using mobile phase A to a final concentration of 0.25% (w/v), filtered through 0.2 µm syringe filters and 20 µL applied to a TSK G2000 SW separating column (600 mm x 7.5 mm I.D.) connected to a TSKGEL SW guard column (50 mm x 7.5 mm I.D., Smyth and FitzGerald, 1997). Separation was by isocratic elution with a mobile phase of 0.1% TFA in 30% acetonitrile at a flow rate of 1.0 mL min^{-1}. Detector response was monitored at 214 nm. A calibration curve was prepared from the average retention times of standard proteins and peptides (0.25 g $(100 mL)^{-1}$) (Smyth and FitzGerald,

1998). The void volume (*V*o) was estimated with BSA (67500 Da), and the total column volume (*V*t) was estimated with L-tyrosine HCl (218 Da).

3.10 IDENTIFICATION OF ANTIOXIDANT PEPTIDES BY UPLC/MS

Samples were diluted in HPLC grade water containing 0.1% (v/v) formic acid. Samples (250 µg) were filtered through a 0.2 µm syringe filter prior to loading onto a BEH C18 column, 1.0 x 150 mm, 1.7 µm and BEH Shield RP18 VanGuard Pre-column, 1.7 µm, 2.1 x 5 mm (Waters, Dublin, Ireland). Peptides were separated by gradient at a flow rate of 0.3 ml/min. Mobile phase A consisted of water, 0.1 % (v/v) formic acid while mobile phase B consisted of 80% (v/v) acetonitrile, 0.1% (v/v) formic acid. Separation was performed employing a linear gradient from 0% to 80% over 45 min. The MS and tandem MS experiments were controlled by MicroTOF control software (version 2.3, Buker Daltonics). Full scans were performed over a *m/z* range of 50-2500. Tandem MS determination was carried out with five automatically selected precursor ions present in the MS scan. Electrospray conditions were as follows: capillary temperature, 190˚C, dry gas (N_2) flow, 8.0 L/min, nebulizer, 1.0 Bar. All MS/MS spectra were initially analysed using Data Analysis software (version 4.0, Bruker Daltonics) and Biotools (version 3.1, Bruker Daltonics). Data was then searched against the SwissProt protein database.

3.11 STATISTICAL ANALYSIS

ORAC data are presented as means ± SD. Statistical analysis was carried out using statistical package Statgraphics Centurion XVI (StatPoint Technologies Inc., USA, 2010) program and significance of each group was verified with the analysis of the One-way ANOVA followed by the Tuckey test at $p < 0.05$.

4. RESULTS AND DISCUSSION

It is widely recognized that proteins are a source of BAPs. Food-derived BAPs have been identified from milk, egg, meat and fish as well as from soy and wheat proteins (Lee *et al.*; 2006; Migueland Aleixandre, 2006; Vercruysse *et al.*, 2005; Nagai *et al.*, 2006; Kodera and Nio, 2006; Motoi and Kodama, 2003). At present, milk proteins are the most extensively studied source of bioactive peptides. Casein hydrolysates, mainly derived from α_{s1}- and β-casein, have been shown to display antioxidant properties (Korhonen and Pihlanto, 2003), free radical-scavenging activities and the ability to inhibit enzymatic and non-enzymatic lipid

peroxidation (Suetsuna *et al.*, 2000; Rival *et al.*, 2001; Srinivas and Prakash, 2010). The objective of the current study was to evaluate the antioxidant activity of bovine caseinate hydrolysates generated using *Cynara cardunculus* L. and *Ficus carica* L. - derived proteinases. The classical approach to produce bioactive peptides is enzymatic hydrolysis of whole food proteins. Many factors affect the enzymatic hydrolysis of proteins, among them enzyme and substrate concentration. Depending on the specificity of the enzyme, environmental conditions and extent of hydrolysis, a wide variety of peptides can be generated. Hydrolysates may possess better functional properties than the original proteins (Panyam and Kilara, 1996). Sodium caseinate (Na-CN) and β-casein (β-CN) were chosen as model substrates since they are commercially available in large quantities and at reasonable cost. Digestion of NaCN with *Cynara cardunculus* L. proteinase resulted in low DH presumably due to the high specificity of enzymatic activity. The specificity of the proteinase used for hydrolysis, in fact, affects both the type of peptides produced, and the extent of hydrolysis achieved, due to the number and location of sites available for cleavage (Panyam and Kilara, 1996). After several trials, a low DH (1.37%) was also found at high E:S ratio (1:10) and time of hydrolysis increased up to 480 min (Fig. 1).

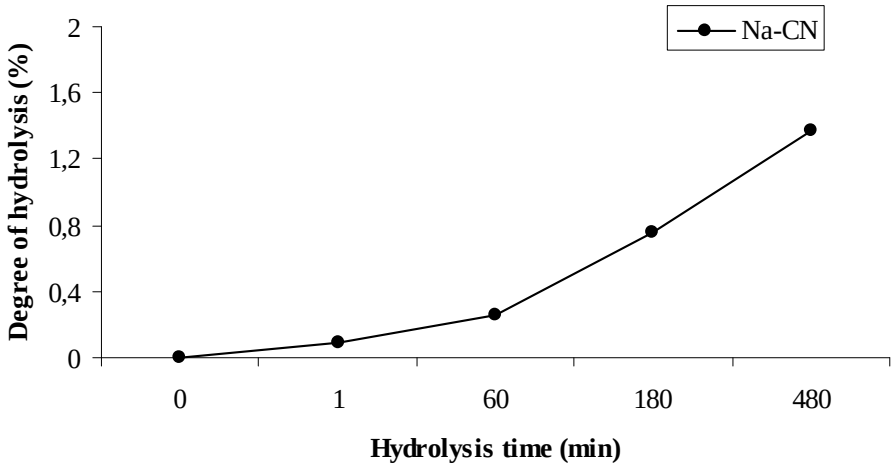

Fig. 1 Degree of hydrolysis (DH, %) of bovine sodium caseinate (Na-CN) hydrolysates obtained with *Cynara cardunculus* L. protease (Enzyme:Substrate = 1:10).

The SDS-PAGE profile of the generated Na-CN hydrolysates was in accordance with the observed DH (Fig. 2). After 1 min and for all the duration of enzymatic reaction, sodium caseinate hydrolysates eluted in 4 major bands at 29, 24, 20 and 14.2 kDa with over minor bands between 14.2 and 6.5 kDa (Fig. 2, lanes 4-7).

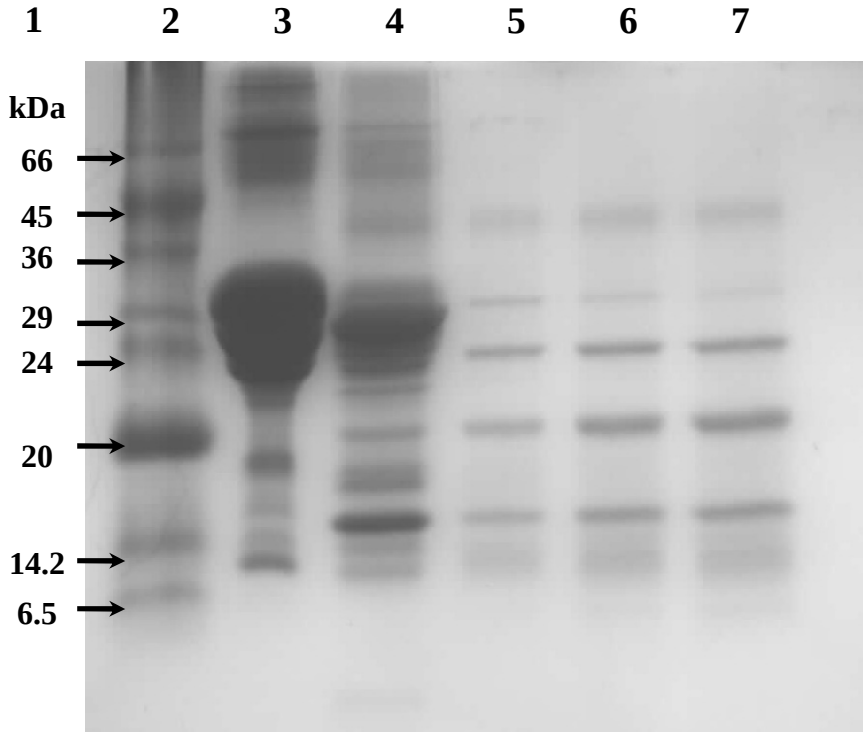

Fig. 2 SDS-PAGE electrophoretograms illustrating the degradation of bovine sodium caseinate (Na-CN) by the crude extract of *Cynara cardunculus* (CC) (Enzyme:Substrate ratio = 1:10). Lane 1 contains molecular weight proteins markers. Lanes 2 contains the intact sodium caseinate. Lanes 3-7 correspond to the hydrolysis of NaCN at different times of incubation: (3) 1 min; (4) 60 min; (5) 180 min; (6) 480 min.

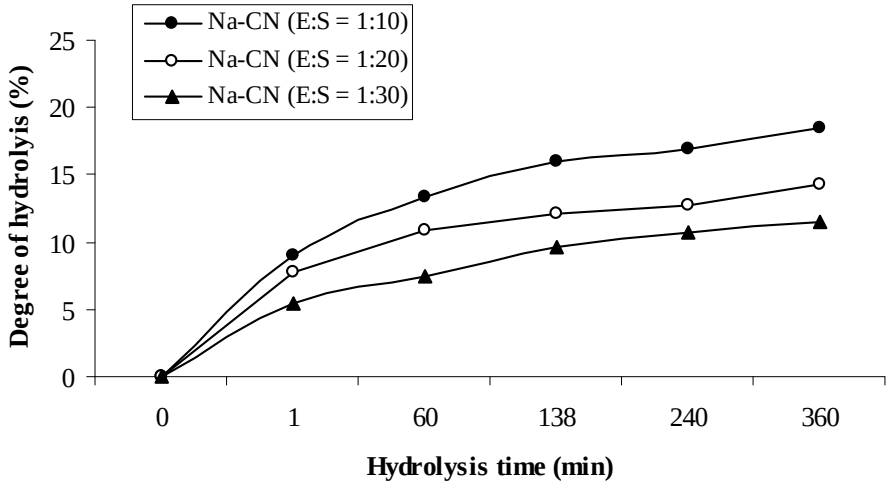

Fig. 3 Degree of hydrolysis (DH, %) of bovine sodium caseinate (Na-CN) hydrolysates obtained with *Ficus carica* L. latex protease (Enzime:Substrate (E: S) = 1:10, 1:20, 1:30).

Therefore, improvements in digesting Na-CN were not observed as time elapsed. On the basis of this result, it was concluded that *Cynara cardunculus* extract, due to its specificity and low

DH, could not generated peptides/hydrolysates with better functional properties than the original protein. Hence, *Cynara cardunculus* L. was excluded from the antioxidant characterization of its peptides/hydrolysates.

On the other hand, the hydrolysis of Na-CN and β-CN allowed analysis of enzymatic action of *Ficus carica* latex. The enzymatic hydrolysis reaction was monitored over 6 h and 4 h for Na-CN and β-casein, respectively. Initially, different E:S ratios were used to obtain bovine Na-CN hydrolysates having a range of DH values. Thus, after several trials, an E:S ratio of 1:10 was found to give the best results (Fig. 3). The rate of hydrolysis was very rapid initially giving a DH value of 9% in the first minute. After that, hydrolysis increases progressively reaching 18% DH after incubation of 6 h. On the basis of these results, an E:S of 1:10 was chosen for the hydrolysis of β-casein in the next step (Fig. 4).

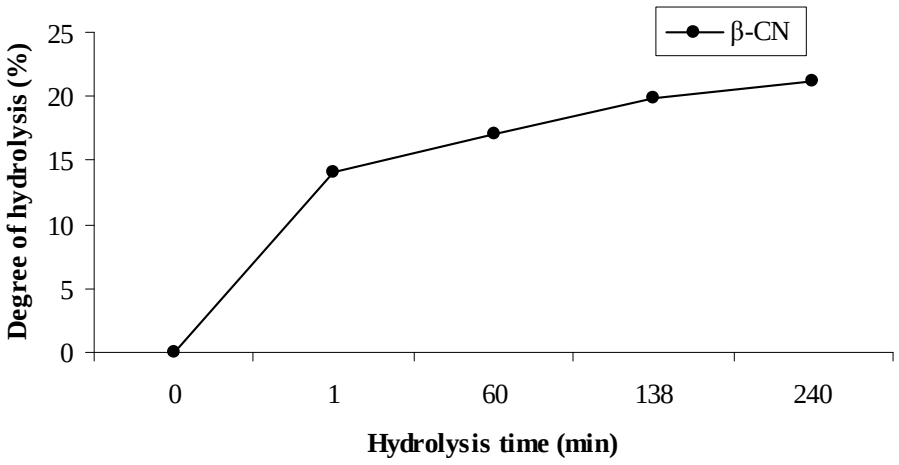

Fig. 4 Degree of hydrolysis (DH, %) of bovine β-casein (β-CN) hydrolysates obtained with *Ficus carica* L. latex protease (Enzyme:Substrate (E.S) = 1:10).

The hydrolysis curve obtained under the experimental conditions, as expected, shows an increase in DH as a function of reaction time. Again, the rate of hydrolysis was very rapid for the first minute (15%), and the DH reached ~ 21% after 4 h hydrolysis (Fig. 4). The difference in the results between Fig. 3 and 4 may be due to differences in the composition of substrates used. In this regard, sodium caseinate contains α_{s1}- α_{s2}- β- and κ-casein, in the proportion of 3:0.8:3:1 by weight (Aoki *et al.*, 1996). Among those proteins, β-casein has more surface-active properties than α_{s1}-casein (Dickinson *et al.*, 1988; Mulvhill and Fox, 1989). Therefore, this property may account for the specificity and concomitant binding affinity of *Ficus carica* protease toward this casein fraction.

Subsequently, the casein hydrolysates were tested for their *in vitro* antioxidant activity using the $ORAC_{FL}$ assay. The $ORAC_{FL}$ assay measures antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C (Cao *et al.*, 1993, 1995). The decrease in relative fluorescence intensity (RFI%), determined using a fluorescent probe (FL, 3',6'-dihydroxyspiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one), is an indication of the extent of oxidative damage from its reaction with the peroxyl radical. Furthermore, the $ORAC_{FL}$ assay is the only method currently available that combines both inhibition time and degree of inhibition in a single quantification (Ou *et al.*, 2001). The fluorescence was recorded every 5 min for 120 min, until the relative fluorescence intensity (RFI%) was less than 5% of the value of the initial reading. Fig. 5 shows the fluorescence intensity decay curves for Na-CN digests (E:S 1:10) at 0, 1 and 360 min of hydrolysis.

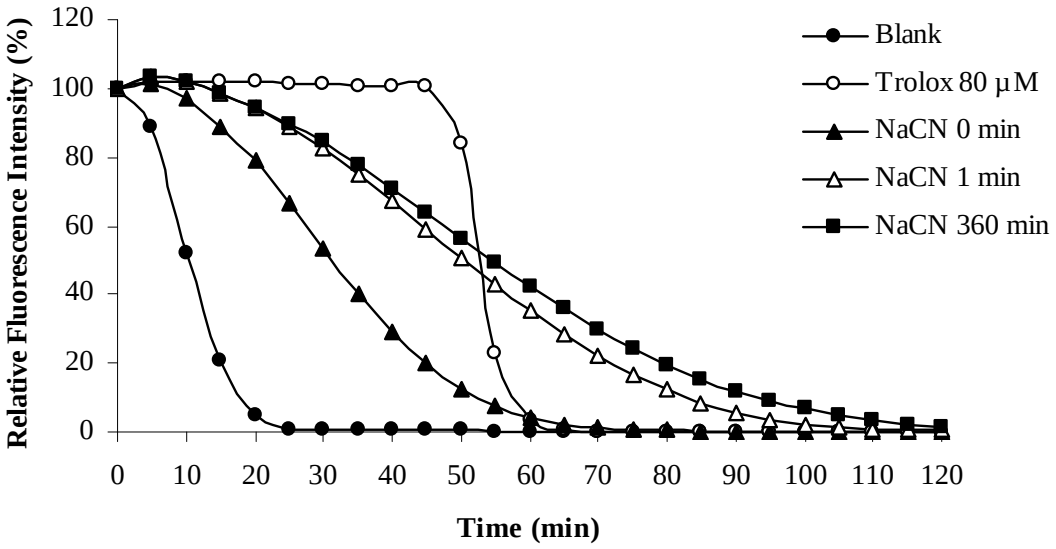

Fig. 5 Fluorescence intensity decay (RFI%) of fluorescein induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence of blank, standard Trolox (80 µM), unhydrolyzed sodium caseinate (Na-CN) (0 min) and its digests (1 and 360 min) at a concentration 0.5 mg/mL.

The results demonstrate how the fluorescence inhibition percentage (RFI%) changes as the reaction takes place. The undigested Na-CN had a lower scavenging activity than its hydrolysates. During the reaction the antioxidant capacity of intact Na-CN decreased gradually, leading to a loss in fluorescence < 5 % after 60 min. On the other hand, the RFI% was greater for the digest at 360 min (DH = 18.49 %) than the digest at 1 min (DH = 9.06%), by preserving fluorescein from AAPH induced oxidation until 105 and 95 min, respectively.

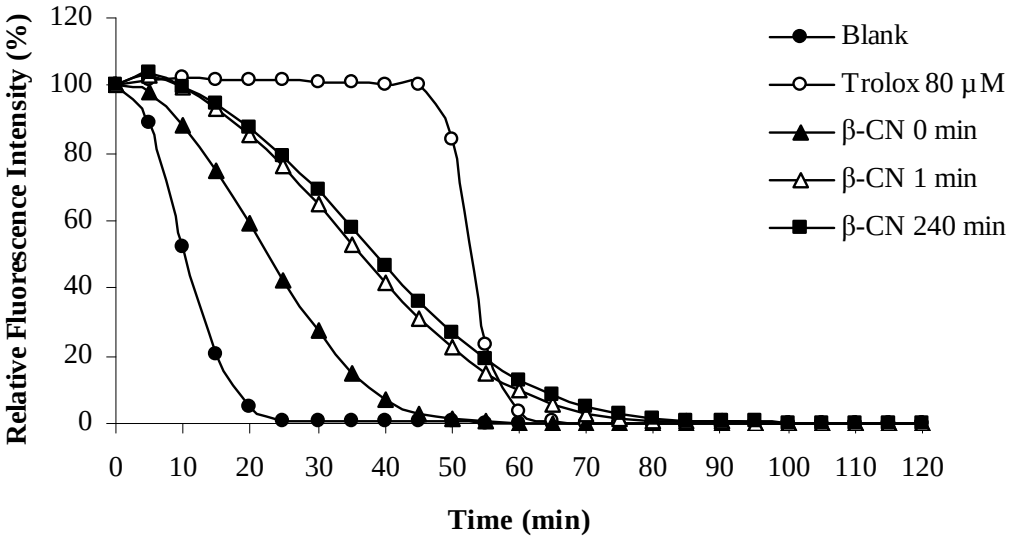

Fig. 6 Fluorescence intensity decay (RFI%) of fluorescein induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence of blank, standard Trolox (80 µM), unhydrolyzed β-casein (β-CN) (0 min) and its digests (1 and 240 min) at a concentration 0.05 mg/mL.

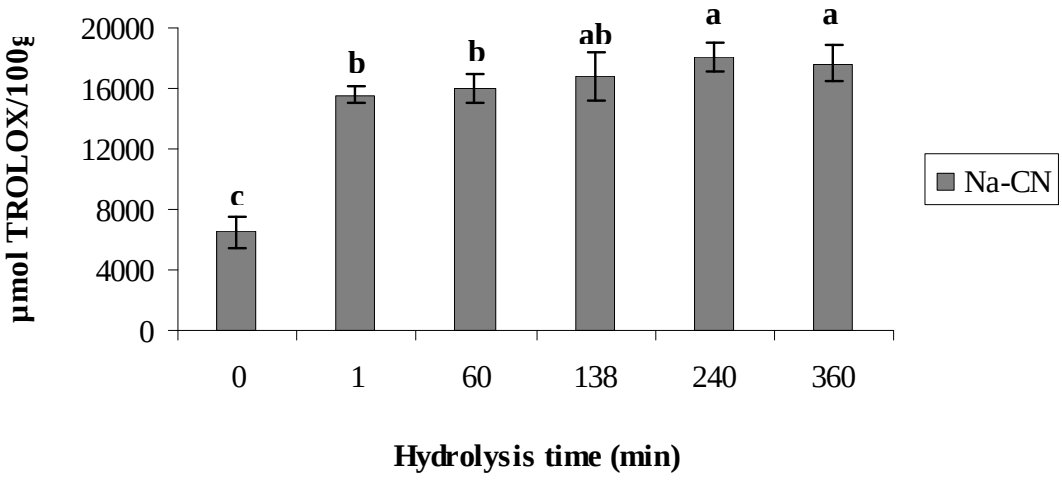

Fig. 7 Oxygen radical absorbance capacity (ORAC) of bovine sodium caseinate (Na-CN) hydrolysates obtained with *Ficus carica* L. latex protease (E:S = 1:10) during *in vitro* hydrolysis. The activity was expressed as µmol Trolox/100g, and the results are plotted as the means ± SD (n = 3). Means with different letters differ significantly ($p > 0.05$). The protein sample tested was at 0.5 mg/mL.

These observations could also be made for the β-CN samples (Fig. 6). However, it must be pointed out that the concentration used (0.05 mg/mL) in the $ORAC_{FL}$ assay for β-CN digests

was ten times lower than the concentration employed for Na-CN hydrolysates. Hence, this difference marks the more potent antioxidant activity of β-casein digests compared to Na-CN hydrolysates. The plateau in RFI (%) was reached at 45 min for nondigested β-casein, whereas it occurred at 70 and 75 min for its hydrolysates at 1 and 240 min incubation, respectively. Hence, a significant difference was observed in the overall ability to scavenge the AAPH radical by bovine sodium caseinate and β-casein hydrolysates (Fig. 5 and 6). A different antioxidant activity is further demonstrated by Na-CN and β-CN samples in Fig. 7 and 8, confirming the previous fluorescence intensity decay curves (Fig. 5 and 6). These samples exhibited strong dose-dependent antioxidant activity. The values of these antioxidant activity indexes, in fact, increased rapidly at low concentrations of Na-CN (0.5 mg/mL) and especially β-CN (0.05 mg/mL), suggesting that both had high antioxidant efficiency in dilute solution. These results agree with those of Zhu *et al.* (2008), who found that hydrolyzed zein was capable of antioxidant activity at low concentrations (< 5 mg/mL). Furthermore, the use of dilute samples was necessary since the ORAC assay is extremely sensitive (Huang *et al.*, 2002). The ORAC values of bovine Na-CN hydrolysates (E:S = 1:10) at 0.5 mg/mL concentration ranged from 6506 to 18081 µmol Trolox/100 g of protein (Fig. 7).

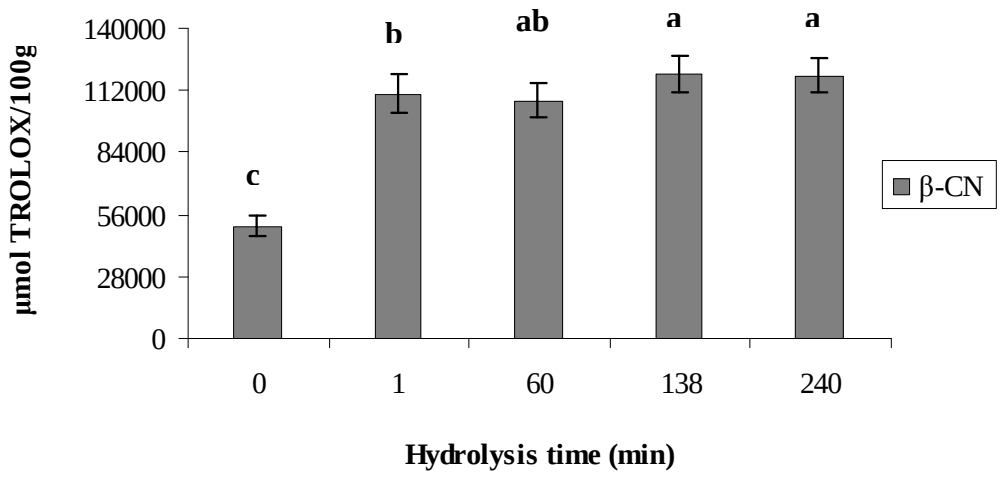

Fig. 8 Oxygen radical absorbance capacity (ORAC) of bovine β-casein (β-CN) hydrolysates obtained with *Ficus carica* L. latex (E:S = 1:10) during *in vitro* hydrolysis. The activity was expressed as µmol Trolox/100g, and the results are plotted as the means ± SD (n = 3). Means with different letters differ significantly ($p > 0.05$). The protein sample tested was 0.05 mg/mL.

As expected, the scavenging activity of the unhydrolysed Na-CN (6506 µmol Trolox/100 g) was significantly lower than the hydrolysed protein. It is well documented that hydrolysates

generally possess higher biofunctional properties than the original proteins (Panyam and Kilara, 1996). Increasing the incubation time with *Ficus carica* L. protease, i.e., from 1 to 360 min, led to negligible increases of antioxidant activity. In particular, no significant difference in antioxidant activity was observed over the next 240 min. However, in comparison to β-casein digests, the effect of Na-CN hydrolysates to scavenge AAPH radical was significantly lower (Fig. 8). The β-CN samples tested in the $ORAC_{FL}$ assay, although at lower concentration (0.05 mg/mL), had a higher antioxidant activity than the Na-CN samples (0.5 mg/mL). The β-CN hydrolysate $ORAC_{FL}$ values varied from 50722 to 118977 μmol Trolox/100 g of protein. The capability to scavenge the AAPH radical was also detectable in nondigested β-CN, whose antioxidant activity has been linked mainly to the five serine-phosphate residues located in the N-terminal region of the molecule (Schmelzer *et al.*, 2007). As described previously, caseins display natural scavenging activity due to their polar domains that contain phosphoseryl residues. Sequential digestion of β-casein with *Ficus carica* L. protease, from 0 to 240 min, produced hydrolysates with higher antioxidant properties than the original protein. The highest mean values were found at 138 and 240 min with 118977 and 118788 μmol Trolox/100 g of protein. These values were higher when compared with those found in a peptic hydrolysate of crude egg protein (0.381 μmol Trolox/mg of protein, Dávalos *et al.*, 2004) or in an ovine α_{s2}-casein hydrolysate obtained with porcine pepsin (0.78 μmol Trolox/mg of protein, López-Expósito *et al.*, 2007). However, this scavenging activity was lower than that reported for a hydrolysate from bovine κ-casein by porcine pepsin (7.07 μmol Trolox/mg of protein, López-Expósito *et al.*, 2007) and for α-lactalbumin digests by Corolase PP and chymotripsin, respectively (2.954 and 2.528 μmol Trolox/mg protein, Hernández-Ledesma *et al.*, 2005). Overall, the general increase of antioxidant activity observed for bovine Na-CN and β-casein digests can be attributed to various factors. As suggested by Zhu *et al.* (2008), with the increase of DH, electron-dense amino acid side chain groups, i.e., polar or charged moieties, become more exposed. Furthermore, an increased availability of free amino acids during digestion provides an additional source of protons and electrons to maintain a high redox potential. These physiochemical changes could also explain the enhanced radical scavenging capacity of bovine Na-CN and β-casein digests. These results seem to support the concept that, as the antioxidant peptides present in these bovine casein hydrolysates are of a different nature, they have different inhibition kinetics and scavenging activities. Moreover, these different scavenging patterns could be related to the structure (Saito *et al.*, 2003) and configuration of peptides produced at different stages as well as to differences in their size, molecular weight

(Li *et al.*, 2008) and amino acid sequence (Chen *et al.*, 1996; 1998). Specially, peptides size is well known to be a significant factor in the overall antioxidant activity of hydrolyzed proteins. Gel permeation HPLC was therefore carried out to better understand the molecular mass distribution of β-CN hydrolysates obtained with *Ficus carica* latex. The molecular mass distribution is summarised in Table 1. Gel permeation HPLC showed that after 1 minute of hydrolysis, β-casein hydrolysates had approximately 52% more peptide material < 0.5 kDa than the intact casein (0 min). The β-CN hydrolysate only had 0.4% of peptide material > 10 kDa after 1 minute hydrolysis. This demonstrates that the addition of *Ficus carica* protease to β-CN produces a rapid hydrolysis in the first minute, as previously observed in the DH curve (Fig. 5). After 60 min incubation, the hydrolysate again had much less peptide material in the molecular mass ranges 2-1 kDa, 2-5 kDa, 5-10 kDa and >10 kDa. Consequently, these hydrolysates had more peptide material 1-0.5 kDa and < 0.5 kDa. For example, the 60 min hydrolysate had 5% more material < 500 Da than the 1 min hydrolysate. After 138 and 240 min, the percentage of the peptide material < 500 Da in the hydrolysates was much higher than the first hour of enzymatic reaction. For instance, the hydrolysate produced at 138 min had a DH of 19.94 % while 0.12 % of the peptide material was > 10 kDa, 0.44, 2.38, 7.78 and 27.29 % of peptide material was between 5-10, 2-5, 2-1 and 1-0.5 kDa, respectively.

Table 1 Molecular mass distribution (determined by gel permeation HPLC) of unhydrolyzed β-casein (0 min) and its hydrolysates after 1, 60, 138, 240 min incubation with *Ficus carica* L.

Hydrolysis time (min)	Molecular mass distribution (kDa)					
	<10	**5-10**	**2-5**	**2-1**	**1-0.5**	**<0.5**
0	97.61	1.17	0.6	0.23	0.14	0.25
1	0.4	0.61	3.33	12.61	30.36	52.69
60	0.11	0.42	2.43	9.23	29.42	58.4
138	0.12	0.44	2.38	7.78	27.29	61.98
240	0.15	0.47	2.34	6.91	25.24	64.88

The hydrolysate obtained at 240 min showed a DH of 21.24 % whereas 64.88% of peptide material was < 500 Da. Therefore, a strong relationship was observable between hydrolysate

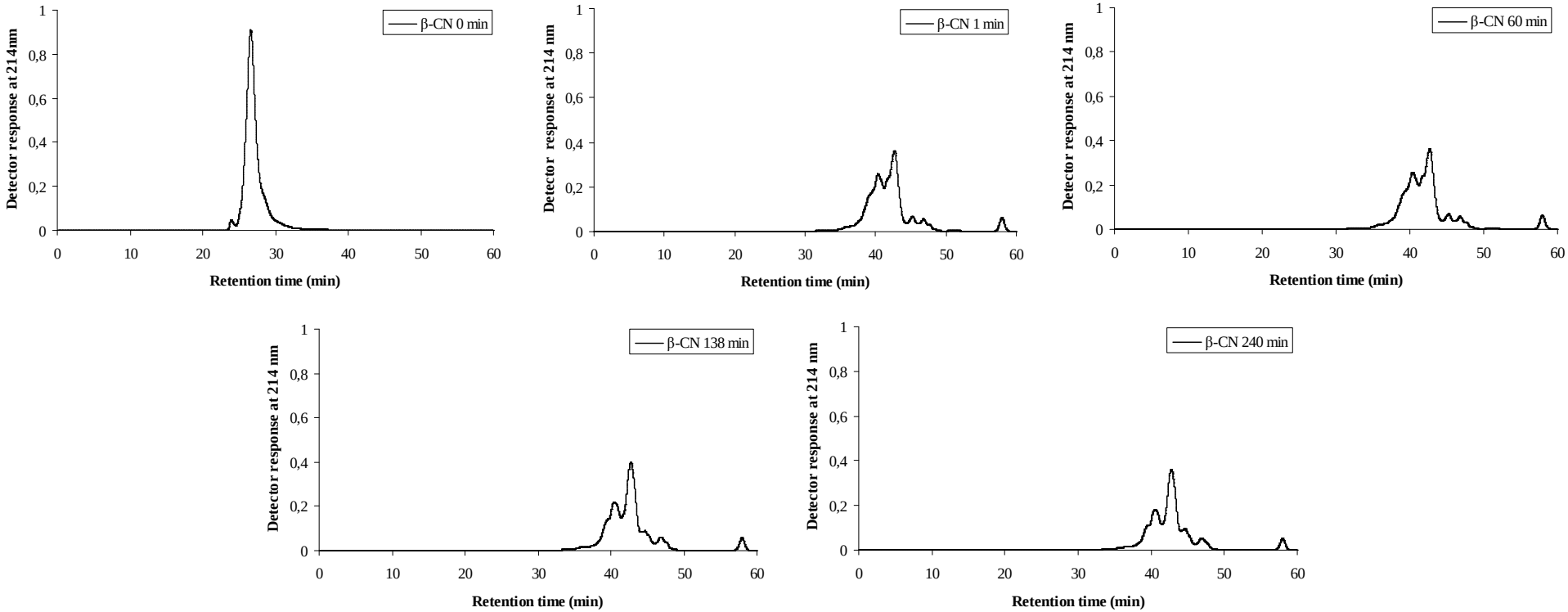

Fig. 9 Gel permeation HPLC profiles of unhydrolyzed β-casein (β-CN) (0 min) and its hydrolysates after 1, 60, 138, 240 min incubation with *Ficus carica* L.

DH and the apparent peptide molecular mass distribution (Fig. 4 and Table 1). The molecular mass profiles are showed in Fig. 9. These chromatograms emphasise the previous observations showing β-casein breakdown during *in vitro* hydrolysis. Overall, this result indicates that the β-casein hydrolysates contain a mixture of low molecular mass peptides. Many studies report that low molecular weight peptides showed high antioxidant activity (Rajapakse *et al.*, 2005). For instance, Peña-Ramos and Xiong (2002) described that short peptides (MW 350-1500 Da) were responsible for the bulk of antioxidant activity in commercial whey protein hydrolysates in a liposome oxidizing system. Wu *et al.* (2003) demonstrated that a peptide from mackerel protein hydrolysate with MW of approximately 1400 Da possessed stronger antioxidant activity than higher MW peptides.

Table 2 Peptide sequences identified after 240 min incubation of β-casein preparation with *Ficus carica* protese at 37°C.

No	β-casein fragment	Peptide sequence	*m /z* measured	Charge	Mr calculated	Mr equivalent (for multicharged)
1	f 47-53	(Q) DKIHPFA (Q)	414.227	2	827.441	827.454
2	f 60-69	(V) YPFPGPIPNS (L)	544.778	2	1088.541	1088.556
3	f 135-140	(H) LPLPLL (Q)	664.4523	1	665.46	-
4	f 146-154	(H) QPHQPLPPT (V)	507.773	2	1014.537	1014.546
5	f 169-176	(S) KVLPVPQK (A)	454.296	2	908.593	907.592
6	f 178-183	(A) VPYPQR (D)	380.212	2	759.415	759.424
7	f 184-188	(R) DMPIQ (A)	603.2902	1	603.281	-
8	f 203-209	(R) GPFPIIV (-)	742.447	1	742.45	-

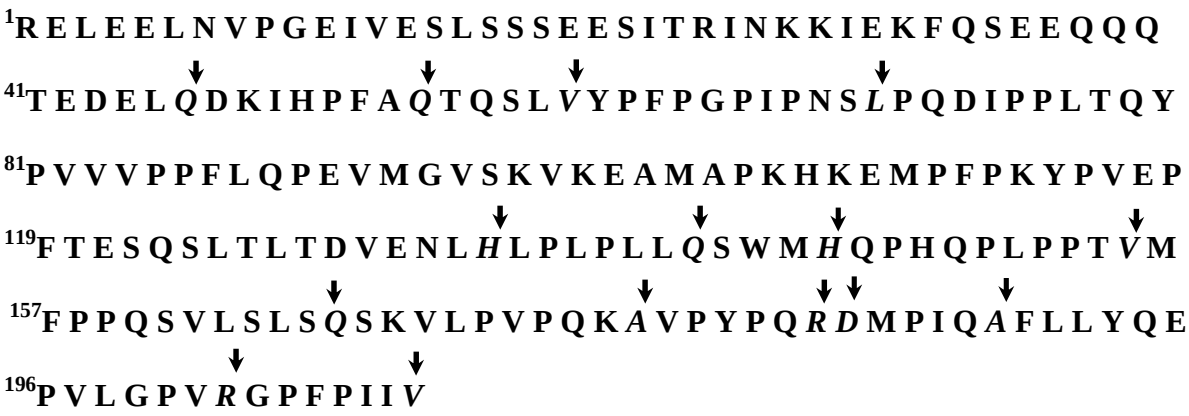

Fig. 10 Theoretically expected cleavage sites for *Ficus carica* protease on bovine β-casein along with the actual cleavages observed on incubation of the β-casein preparation with *Ficus carica* protease both at 37°C. Arrows represent the theoretical cleavage sites, residues in italics represent the cleavages observed at 37°C.

Other authors (Chen *et al.*, 1995) reported that six peptides isolated from a soybean protein hydrolysate with 5–16 amino acid residues were highly effective in inhibiting the autoxidation of linoleic acid. In the present study, the final digest of β-CN (240 min) had 64.88% of peptide material with MW < 500 Da. Amino acid composition and sequence also affect biopeptide activity (Chen *et al.*, 1998; Pihlanto, 2000), i.e., antioxidant activity. During hydrolysis proteinase specificity is key as it dictates the amino acid sequence of the resultant peptides released and thus their biofunctional properties (Murray and FitzGerald, 2007). Therefore, the next step was to study peptides responsible for the antioxidant activity detected in the β-casein hydrolysates as well as the proteinase specificity of *Ficus carica* by UPLC/MS. The peptide sequences, experimental mass and calculated mass of multicharged ions of β-casein hydrolysate, after 240 min incubation with *Ficus carica* protease, are given in Table 2. The primary structure of β-casein and the cleavages observed are reported in Fig. 10. *Ficus carica* protease showed a broad specificity toward neutral and basic amino acids, i.e., Gln, Val, Leu, His Ser, Ala, Asn and Arg. The peptide sequences observed in the 240 min hydrolysate corresponded mainly to the C-terminal region of β-casein. Theoretically a total of 15 different cleavage sites exist in β-casein (Fig. 10). However, in our proteolysis conditions, the fragment 70-134 was not cleaved and was still present after 240 min of hydrolysis. According to Léonil *et al*. (1988) β-casein disappearance is strongly associated with the stable fragments 100-105, 170-176, and 177-183. Thus, these observations may also explain the resistance of this fragment to the proteolytic action of *Ficus carica*. Theoretically, the action of *Ficus carica* protease on β-casein should release one tetra-peptide SWMH (f142-145), one penta-peptide, TQSLV (f55–59), one esa-peptide, DMPIQA (f185–190), and three epta-peptides, LPLPLLQ (f135-141), VPYPQRD (f178-f184) and GPFPIIV (f203-209). As reported by Chen *et al*. (1996; 1998), antioxidant peptides contain 5-16 amino acid residues. In this regard, peptides derived from the hydrolysis of *Ficus carica* proteinase on β-casein had > 5 amino acid residues. In addition, the antioxidant activity of these peptides could be related to their composition, structure and hydrophobicity. Some studies have established that Tyr, Lys, Cys, His and hydrophobic amino acids, i.e. Trp, Met, Pro, Ile, Leu, Val, Gly, Ala, are connected to peptide antioxidant potency (Peña-Ramos *et al.*, 2004; Wang and De Meija, 2005, Pihlanto, 2006). Hydrolysis can also increase or decrease this hydrophobicity which mostly depends on the primary sequence of the substrate protein (Calderón de la Barca *et al.*, 2000). In this regard, bovine β-casein is significantly more hydrophobic than the other caseins (Swaisgood, 2003) because of its large hydrophobic C-terminal domain its digestion can release even more hydrophobic fragments (Léonil *et al.*, 1988). Consequently, peptide

sequences, identified after 240 min incubation of β-casein with *Ficus carica* proteinase, possessed high levels of hydrophobic amino acids. Therefore, a structure-function relationship between the amino acid sequence and the antioxidant capacity may be postulated. For example, the fragments DKIHPFA (f47-53), LPLPLL (f135-140) and GPFPIIV (f203-209) comprised His, Leu, Pro residues and other hydrophobic amino acids, i.e., Ala, Gly, Phe, Val, which seemed to be linked with antioxidant activity. These observations are similar to those one of Chen *et al*. (1995; 1996), who showed that antioxidant activity was highly dependent on the His-His segment in Leu-Leu-Pro-His-His sequence. The antioxidant activity of this peptide was decreased by removing a His residue from the C-terminus. Moreover, the antioxidant activity of VPYPQR (f178-183) may be linked to the Tyr residue, which is potent hydrogen donor. Amino acids with aromatic residues can donate protons to electron deficient radicals (Rajapakse *et al*., 2005). This is in agreement with Rival *et al*. (2001a), who attributed the antioxidant activity of the β-casein fragment AVPYPQR (f177-183) to its Tyr residue. Finally, the β-casein derived peptide KVLPVPQK (f169-176) showed a discrepancy between the experimental and the calculated mass (almost 1 Da), therefore we cannot be confident of its existence in the hydrolysate. However, Rival *et al*. (2001b) obtained the same peptide fragment from a tryptic β-casein digest, which was found to be the most potent inhibitor of lipoxygenase activity. Even though release of the f169-176 was unexpected, the authors explained its formation by atypical cleavage due to chymotrypsin activity present in the trypsin preparation (Visser *et al*., 1995), to unusual enzyme activity, or even to unknown origin (Jones *et al*., 1991). In this regard, it might be asserted that *Ficus carica* latex contained an atypical proteinase activity, which hydrolyzed the Ser-Lys bond.

Overall, from the results obtained, it was observed that degree of hydrolysis, specificity of *Ficus carica* proteinase, size, composition and hydrophobicity of peptides were some of the factors that affected the antioxidant activity of hydrolyzed bovine casein. Therefore, these findings can be useful to explain the results obtained in the present study.

5. CONCLUSIONS

In summary, this chapter reports for the first time the antioxidant activity of *Ficus carica* L. hydrolysates of bovine casein. The ability of Na-CN and β-CN hydrolysates to have antioxidant activity appears to be related to the nature and composition of peptides produced at different stages of hydrolysis. The peptide sequences, identified in β-casein hydrolysates, seem to confirm their antioxidant activity for the high levels of His, Leu, Pro residues and

other hydrophobic amino acids as well as the broad specificity of *Ficus carica* proteinase. These peptides need to be investigated for their effects on quality, taste and shelf-life of food products. Subsequently, further studies need to be carried out to assess the biological benefits, i.e., antioxidant, ACE-inhibitory, and immunomodulatory activity, in animal and human models in order to enhance current knowledge of plant proteinase release of bioactive peptides from food proteins as a source of natural antioxidants.

6. REFERENCES

Adler-Nissen, J. (1979a) 'Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid', *Journal of Agricultural and Food Chemistry*, 27, 1256-1262.

Adler-Nissen, J. (1979b) 'Determination of the degree of hydrolysis of food protein hydrolysates by the trinitrobenzenesulfonic acid method', Novo Enzyme Products Ltd., Technical Information, TN/004.

Aoki, T., Uehara, T., Yonemasu, A. and El-Din, M. Z. (1996) 'Response surface analyses of the effects of calcium and phosphate on the formation and properties of casein micelles in artificial micelles system', *Journal of Agricultural and Food Chemistry*, 44(5), 1230-1235.

Allen, J. C. and Wrieden, W. L. (1982) 'Influence of milk proteins on lipid oxidation in aqueous emulsion. I. Casein, whey protein and α-lactalbumin', *Journal of Dairy Research*, 49, 239-248.

Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J. and Baeyens-Volant, D. (2011) 'Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from *Ficus carica* latex', *Phytochemistry*, 72, 1718-1731.

Butterfield, D. A. (2002) 'Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review', *Free Radical Research*, 36, 1307-1313.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, 72, 248-254.

Brantl, V., Teschemacher, A., Henschen, A. and Lottspeich, F. (1979) 'Novel opioid peptides derived from casein (β-casomorphins)', *Hoppe Seyler's Zeitschrift fur Physiologische Chemie*, 360(9), 1211-1216.

Bruno, M. A., Pardo, M. F., Caffini, N. O. and López, L. M. I. (2002) 'Purification of a New Endopeptidase Isolated from Fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*)', *Acta Farmacéutica Bonaerense*, 21 (1), 51-56.

Calderón de la Barca, A. M., Ruiz-Salazar, R. A. and Jara-Marini, M. E. (2000) 'Enzymatic hydrolysis of soy protein to improve its amino acid composition and functional properties', *Journal of Food Science*, 65, 246-53.

Cao, G., Alessio, H. M. and Culter, R. (1993) 'Oxygen-radical absorbance capacity assay for antioxidants', *Free Radical Biology and Medicine*, 14, 303-311.

Cao, G., Verdon, C. P., Wu, A. H. B., Wang, H. and Prior, R. L. (1995) 'Automated assay of oxygen radical absorbance capacity with the COBAS FARA II', *Clinical Chemistry*, 41, 1738-1744.

Cervato, G., Cazzola, R. and Cestaro, B. (1999) 'Studies on the antioxidant activity of milk caseins', *International Journal of Food Science and Nutrition*, 50, 291-294.

Chen, H.-M., Muramoto, K. and Yamauchi, F. (1995) 'Structural analysis of antioxidative peptides from soybean-conglycinin', *Journal of Agricultural and Food Chemistry*, 43, 574-578.

Chen, H.-M., Muramoto, K., Yamauchi, F. and Nokihara, K. (1996) 'Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein', *Journal of Agricultural and Food Chemistry*, 44, 2619-2623.

Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K. and Nokihara, K. (1998) 'Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein', *Journal of Agricultural and Food Chemistry*, 46, 49-53.

Chiba, H., Tani, F. and Yoshikawa, M. (1989) 'Opioid antagonist peptides derived from κ-casein' *Journal of Dairy Research*, 56(3), 363-366.

Chowienczyk, P. J., Brett, S. E., Gopaul, N. K., Meeking, D., Marchetti, M., Russell-Jones, D. L., Anggard, E. E. and Ritter, J. M. (2000) 'Oral treatment with an antioxidant (raxofelast) reduces oxidative stress and improves endothelial function in men with type II diabetes', *Diabetologia*, 43, 974-977.

Colbert, L. B. and Decker, E. A. (1991) 'Antioxidant activity of an ultrafiltration permeate from acid whey', *Journal of Food Science*, 56, 1248-1250.

Coste, M., Rochet, V., Leonil, J., Molle, D., Bouhallab, S. and Tome, D. (1992) 'Identification of C-terminal peptides from bovine β-casein that enhance proliferation of rat lymphocytes', *Immunology Letters*, 33(1), 41-46.

Cross, K. J., Huq, N.L., Palamara, J.E., Perich, J.W. and Reynolds, E.C. (2005) 'Physicochemical characterization of casein phosphopeptideamorphous calcium phosphate nanocomplexes', *Journal of Biological Chemistry*, 280, 15362-15369.

Dávalos, A., Miguel, M., Bartalomé, B. and López-Fandiño, R. (2004) 'Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis', *Journal of food protection*, 67(9), 1939-1944.

Devaraj, K. B., Kumar, P. R. and Prakash, V. (2008) 'Purification, Characterization, and Solvent Induced Thermal Stabilization of Ficin from *Ficus carica*', *Journal of Agricultural and Food Chemistry*, 56, 11417-11423.

Diaz, M. and Decker, E. A. (2004) 'Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef', *Journal of Agricultural and Food Chemistry*, 52, 8208-8213.

Diaz, M., Dunn, C. M., McClements, D. J. and Decker, E. A. (2003) 'Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions', *Journal of Agricultural and Food Chemistry*, 51, 2365-2370.

Dickinson, E., Rolfe, S. E. and Dalgleish, D. G. (1988) 'Competitive adsorption of α_{s1}-casein, and β-casein in oil-in-water emulsions', *Food Hydrocolloids*, 2, 397-405.

Fiat, A. -M. and Jollès, P. (1989) 'Caseins of various origin and biologically active casein peptides and oligosaccharides: structural and physiological aspects', *Molecular and Cellular Biochemistry*, 87, 5-30.

FitzGerald, R. J. (1998) 'Potential uses of caseinophosphopetides', *International Dairy Journal*, 8, 451-457.

FitzGerald, R. J. and Meisel, H. (2003) 'Milk protein hydrolysates and bioactive peptides', in Fox, P. F. and McSweeney, P. L. H., eds., *Advanced Dairy Chemistry Volume 1: Proteins*, 3rd ed., New York, NY, USA: Kluwer Academic/Plenum Publishers, 675-698.

FitzGerald, R. J., Murray, B. A. and Walsh, D. J. (2004) 'Hypotensive peptides from milk proteins', *Journal of Nutrition*, 134, S980-S988.

Fitzgerald, R. J. and Murray, B. A. (2006) 'Bioactive peptides and lactic fermentations', *International Journal Dairy Technology*, 59, 118-125.

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A. and DiCagno, R. (2002) 'Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing', *Critical Reviews in Food Science and Nutrition*, 42, 223-239.

Hagen, T. M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C. M., Ames, B. N. and Chisari, F. V. (1994) 'Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma', *Proceedings of the National Academy of Sciences of the United States of America*, 91, 12808-12812.

Halliwell, B., Murcia, M. A., Chirico, S. and Arumoma, O.I. (1995) 'Free radicals and antioxidants in food and in vivo: what they do and how they work', *Critical Reviews in Food Science and Nutrition*, 35, 7-20.

Halliwell, B. (2000) 'Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward?', *Cardiovascular Research*, 47, 410-418.

Halliwell, B. (2001) 'Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment', *Drugs Aging*, 18, 479-484.

Halliwell, B. (2002a) 'Vitamin E and the treatment and prevention of diabetes: a case for a controlled clinical trial', *Singapore Medical Journal*, 43, 479-484.

Halliwell, B. (2002b) 'Effect of diet on cancer development: is oxidative DNA damage a biomarker?', *Free Radical Biology and Medicine*, 32, 968-974.

Hambræus, L. (1992) 'Nutritional aspects of milk proteins', in Fox, P. F. and McSweeney, P. L. H., eds., *Advanced Dairy Chemistry Volume 1: Proteins*, 2nd ed., New York, NY, USA: Kluwer Academic/Plenum Publishers, 57-490.

Hartmann, R. and Meisel, H. (2007) 'Food-derived peptides with biological activity: from research to food applications', *Current Opinion in Biotechnology*, 18, 163-169.

Hernández-Ledesma, B., Dávalos, A., Bartolomé, B. and Amigo, L. (2005) 'Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLCMS', *Journal of Agricultural and Food Chemistry*, 53, 588-593.

Horiguchi, N., Horiguchi, H. and Suzuki, Y. (2005) 'Effect of wheat gluten hydrolysate on the immune system in healthy human subjects', *Bioscience Biotechnology and Biochemistry*, 69, 2445-2449.

Hogan, S., Zhang, L., Li, J., Wang, H. and Zhou, K. (2009) 'Development of antioxidant rich peptides from milk protein by microbial proteinases and analysis of their effects on lipid peroxidation in cooked beef', *Food Chemistry*, 117, 438-443.

Hu, M., McClements, D. J. and Decker, E. A. (2003a) 'Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions', *Journal of Agricultural and Food Chemistry*, 51, 1435-1439.

Hu, M., McClements, D. J. and Decker, E. A. (2003b) 'Lipid oxidation in corn in oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate', *Journal of Agricultural and Food Chemistry*, 51, 1696-1700.

Huang, D., Ou, B., Hampsch-Woodill. M., Flanagan, J. A. and Prior, R. L. (2002) 'High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format', *Journal of Agricultural and Food Chemistry*, 50, 4437-4444.

Ito, N., Fukushima, S. and Tsuda, H. (1985) 'Carcinogenicity and modification of the carcinogenic response by BHA, BHT and other antioxidants', *CRC Critical Reviews in Toxicology*, 15, 109-150.

Jones, D. S., Heerma, W., van Wassenaar, P. D. and Haverkamp, J. (1991) 'Analysis of bovine β-casein tryptic digest by continuous flow fast-atom bombardment mass spectrometry', *Rapid Communication in Mass Spectrometry*, 5, 192-195.

Kansci, G., Genot, C., Meynier, A., Gaucheron, F. and Chobert, J. M. (2004) 'β-Caseinophosphopeptide (f 1-25) confers on β-casein tryptic hydrolysate an antioxidant activity during iron/ascorbate-induced oxidation of liposomes', *Lait*, 84, 449-462.

Kayser, H. and Meisel, H. (1996) 'Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins', *FEBS Letters*, 383(1-2), 18-20.

Kilara, A. and Panyam, D. (2003) 'Peptides from milk proteins and their properties', *Critical Reviews in Food Science and Nutrition*, 43, 607-633.

Kitts, D. D. and Yuan, Y. V. (1992) 'Caseinophosphopeptides and calcium bioavailability', *Trends in Food Science and Technology*, 3, 31-35.

Kitts, D. (1994) 'Bioactive peptides in food: Identification and potential uses', *Canadian Journal of Physiology and Pharmacology*, 72(4), 423-434.

Kitts, D. D. and Weiler, K. (2003) 'Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery', *Current Pharmaceutical Design*, 9, 1309-1323.

Kodera, T. and Nio, N. (2006) 'Identification of an angiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive

effects of hydrolysates in spontaneously hypertensive model rats', *Journal of Food Science*, 71, C164-C173.

Korhonen, H. and Pihlanto, A. (2003a) 'Bioactive peptides: novel applications for milk proteins', *Applied Biotechnology Food Science and Policy*, 1, 133–144.

Korhonen, H. and Pihlanto, A. (2003b) 'Food-derived bioactive peptides-opportunities for designing future foods', *Current Pharmaceutical Design*, 9, 1297-1308.

Korhonen, H. and Pihlanto, A. (2006) 'Bioactive peptides: Production and functionality', *International Dairy Journal*, 16, 945-960.

Korhonen, H. (2009) 'Milk-derived bioactive peptides: from science to applications', *Journal of Functional Food*, I, 177-187.

Kudoh, Y., Matsuda, S., Igoshi, K. and Oki, T. (2001) 'Antioxidative peptide from milk fermented with *Lactobacillus delbrueckii* subsp. bulgaricus IFO13953', *Journal of the Japanese Society for Food Science and Technology*, 48, 44-50.

Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T. and Zilmer, M. (2003) 'Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects', *British Journal of Nutrition*, 90, 449-456.

Lahov, E. and Regelson, W. (1996) 'Antibacterial and immunostimulating casein-derived substances from milk: Caseicidin, isracidin peptides', *Food and Chemical Toxicology*, 34(1), 131-145.

Lajoie, N., Gauthier, S. F. and Pouliot, Y. (2001) 'Improved storage stability of model infant formula by whey peptides fractions', *Journal of Agricultural and Food Chemistry*, 49, 1999-2007.

Lee, N. Y., Cheng, J. T., Enomoto, T. and Nakano, Y. (2006) 'One peptide derived from hen ovotransferrin as pro-drug to inhibit angiotensin converting enzyme', *Journal of Food and Drug Analysis*, 14, 31-35.

Léonil, J., Mollé, D. and Maubois, J. L. (1988) 'Study of the early stages of tryptic hydrolysis of β-casein', *Lait*, 68, 281-294.

Li, X. X., Han, L. J. and Chen, L. J. (2008) 'In vitro antioxidant activity of protein hydrolysates prepared from corn gluten meal', *Journal of the Science of Food and Agriculture*, 88, 1660-1666.

López-Expósito, I., Quirós, A., Amigo, L. and Recio, I. (2007) 'Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides', *Lait*, 87, 241-249.

Lo Piero, A. R., Puglisi, I. and Petrone, G. (2002) 'Characterization of 'Lettucine,'' a serine-like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting', *Journal of Agriculture and Food Chemistry*, 50, 2439-2443.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A. and Klee, W. A. (1983) 'Opioid activities and structures of α-casein-derived exorphins', *Biochemistry*, 22(19), 4567-4573.

Liu, Q., Raina, A., Smith, M., Sayre, L. and Perry, G. (2003) 'Hydroxynonenal, toxic carbonyls, and Alzheimer disease', *Molecular Aspects of Medicine*, 24, 305-313.

Mao, X. Y., Yang, H. Y., Song, J. P., Li, Y. H. and Ren, F. Z. (2007) 'Effect of yak milk casein hydrolysate on Th1/Th2 cytokines production by murine spleen lymphocytes in vitro', *Journal of Agricultural and Food Chemistry*, 55, 638-642.

Maté, J. I., Frankel, E. N. and Krochta, J. M. (1996) 'Whey protein isolate edible coating: Effect on the rancidity process of dry roasted peanuts', *Journal of Agricultural and Food Chemistry*, 44, 1736-1740.

McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H. and Coventry, M. J. (2006) 'Isolation and characterisation of a novel antibacterial peptide from bovine α_{s1}-casein', *International Dairy Journal*, 16, 316-323.

Meisel, H. (1986) 'Chemical characterization and opioid activity of exorphin isolated from *in vivo* digests of casein', *FEBS Letters*, 196, 223-227.

Meisel, H. and Schlimme, E. (1994) 'Inhibitors of angiotensin-converting enzyme derived from bovine casein (casokinins)', in Brantl, V. and Teschemacher, H., eds., *β-casomorphins and Related Peptides: Recent developments*, Weinheim, GERMANY: VCH, 27-33.

Meisel, H. (1998) 'Overview on milk protein-derived peptides', *International Dairy Journal*, 8(5-6), 363-373.

Meisel, H. and Bockelmann. W. (1999) 'Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties', *Antonie van Leeuwenhoek*, 76, 207-215.

Meisel, H. and FitzGerald, R. J. (2003) 'Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects', *Current Pharmaceutical Design*, 9, 1289-1295.

Meisel, H. (2004) 'Multifunctional peptides encrypted in milk proteins' *Biofactors*, 21, 55-61.

Migloire-Samour, D. and Jollès, P. (1988) 'Casein, a pro-hormone with immunomodulating role to the newborn?', *Experientia*, 44(3), 188-193.

Migloire-Samour, D., Floch, F. and Jollès, P. (1989) 'Biologically active casein peptides implicated in immunomodulation', *Journal of Dairy Research*, 56(3), 357-362.

Miguel, M. and Aleixandre, A. (2006) 'Antihypertensive peptides derived from egg proteins', *Journal of Nutrition*, 136, 1457-1460.

Minkiewicz, P., Slangen, C. J., Dziuba, J., Visser, S. and Mioduszewska, H. (2000) 'Identification of peptides obtained via hydrolysis of bovine casein by chymosin using HPLC and mass spectrometer', *Milchwissenschaft*, 55(1), 14-17.

Motoi, H. and Kodama, T. (2003) 'Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides from wheat gliadin hydrolysate', *Nahrung/Food*, 47, 354-358.

Mulvhill, D. M. and Fox, P. F. (1989) 'Caseins: functional properties'. In P. F. Fox, ed., *Developments in dairy chemistry*, 4th ed., London, UK: Elsevier Applied Science Publishers, 131-172.

Murray, B. A. and FitzGerald, R. J. (2007) 'Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production', *Current Pharmaceutical Design*, 13(8), 773-791.

Nagai, T., Suzuki, N., Tanoue, Y., Kai, N. and Nagashima, T. (2007) 'Antioxidant and antihypertensive activities of autolysate and enzymatic hydrolysates from yam (*Dioscorea opposita Thunb.*) ichyoimo tubers', *J Food Agric Environ*, 5, 64-68.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S. and Takano, T. (1995) 'Purification and characterization of angiotensin-I-converting enzyme inhibitors from sour milk', *Journal of Dairy Science*, 78, 777-783.

Nakamura, Y., Yamamoto, N., Sakai, K. and Takano, T. (1995) 'Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin-I converting enzyme', *Journal of Dairy Science*, 78, 1253-1257.

Nongonierma, A. B. and Fitzgerald, R. J. (2012a) 'Biofunctional properties of caseinophosphopeptides in the oral cavity', *Caries Research*, 46(3), 234-267.

Nongonierma, A. B. and Fitzgerald, R. J. (2012b) 'Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase', *Peptides*, 37, 263-272.

Okada, Y. and Okada, M. (1998) 'Scavenging effect of water soluble proteins in broad beans on free radicals and active oxygen species', *Journal of Agricultural and Food Chemistry*, 46, 401-406.

Ou, B., Hampsch-Woodill, M. and Prior, R. L. (2001) 'Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe', *Journal of Agricultural and Food Chemistry*, 49, 4619-4626.

Panyam, D. and Kilara, A. (1996) 'Enhancing the Functionality of Food Proteins by Enzymatic Modification', *Trends in Food Science and Technology*, 7, 120-125.

Park, E. Y., Murakami, H., Mori, T. and Matsumura, Y. (2005) 'Effects of protein and peptide addition on lipid oxidation in powder model system', *Journal of Agricultural and Food Chemistry*, 53, 137-144.

Parthasarathy, S., Santanam, N., Ramachandran, S. and Meilhac, O. (2000) 'Potential role of oxidized lipids and lipoproteins in antioxidant defense', *Free Radical Research*, 33, 197-215.

Peña-Ramos, E. A. and Xiong, Y. L. (2002) 'Antioxidative activity of soy protein hydrolysates in a liposomal system', *Journal of Food Science*, 67, 2952-2956.

Peña-Ramos, E. A. and Xiong, Y. L. (2003) 'Whey and soy protein inhibit lipid oxidation in cooked pork patties', *Meat Science*, 64, 259-263.

Peña-Ramos, E. A., Xiong, Y. L. and Arteaga, G. E. (2004) 'Fractionation and characterization for antioxidant activity of hydrolysed whey protein', *Journal of the Science of Food and Agricultural*, 84, 1908-1918.

Perpetuo, E. A., Juliano, L. and Lebrun, I. (2003) 'Biochemical and pharmacological aspects of two bradykinin-potentiating peptides obtained from tryptic hydrolysis of casein', *Journal of Protein Chemistry*, 22(7-8), 601-606.

Phelan, M., Aherne, A., FitzGerald, R. J. and O'Brien, N. M. (2009a) 'Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status', *International Dairy Journal*, 19, 643-654.

Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J. and O'Brien, N. M. (2009b) 'Potential bioactive effects of casein hydrolysates on human cultured cells', *International Dairy Journal*, 19, 279-285.

Pihlanto-Leppälä, A., Rokka, T. and Korhonen, H. (1998) 'Angiotensin-I-converting enzyme inhibitory peptides derived from bovine milk proteins', *International Dairy Journal*, 8, 325-331.

Pihlanto-Leppala, A. (2000) 'Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory', *Trends Food Science and Technology*, 11, 347-56.

Pihlanto, A. (2006) 'Antioxidative peptides derived from milk proteins', *International Dairy Journal*, 16, 1306-1314.

Pihlanto, A., Akkanen, S. and Korhonen, H. J. (2008) 'ACE-inhibitory and antioxidant properties of potato (*Solanum tuberosum*)', *Food Chemistry*, 109, 104-112.

Rajapakse, N., Mendis, E., Byun, H. G. and Kim, S. K. (2005) 'Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems', *Journal of Nutritional Biochemistry*, 16, 562-569.

Rizzello, C. G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M. D. and Zambonin, P. G. (2005) 'Antibacterial activities of peptides from the water-soluble extracts of Italian cheese varieties', *Journal of Dairy Science*, 88, 2348-2360.

Rival, S. G., Boeriu, C. G. and Wichers, H. J. (2001a) 'Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition', *Journal of Agricultural and Food Chemistry*, 49, 295-302.

Rival, S. G., Fornaroli, S., Boeriu, C. G. and Wichers, H. J. (2001b) 'Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties', *Journal of Agricultural and Food Chemistry*, 49, 287-294.

Saiga, A., Tanabe, S. and Nishimura, T. (2003) 'Antioxidant activity of peptides obtained from porcien myofibrillar proteins by protease treatment', *Journal of Agricultural and Food Chemistry*, 51, 3661-3667.

Saito, K., Jin, D. H., Ogawa, T., Muramoto, K., Hatakeyama, E., Yasuhara, T. and Nokihara, K. (2003) 'Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry', *Journal of Agricultural and Food Chemistry*, 51, 3668-3674.

Sarmadi, B. H and Ismail, A. (2010) 'Antioxidative peptides from food proteins: A review', *Peptides*, 31, 1949-1956.

Schanbacher, F. L., Talhouk, R. S., Murray, F. A., Gherman, L. I. and Willett, L. B. (1998) 'Milk-borne bioactive peptides', *International Dairy Journal*, 8, 393-403.

Schmelzer, C. E. H., Schöps, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R. H. H. and Raith, K. (2007) 'Peptic digestion of β-casein. Time course and fate of possible bioactive peptides', *Journal of Chromatography A*, 1166, 108-115.

Sgarbieri, V. C., Gupte, S. M., Kramer, D. E. and Whitaker, J. R. (1964) 'Ficus enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* lattices', *The Journal of Biological Chemistry*, 239, 2170-2177.

Sidrach, L., García-Cánovas, F., Tudela, J. and Rodríguez-López, J. N. (2005) 'Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A', *Phytochemistry*, 66, 41-49.

Silva, S. V. and Malcata, F. X. (2005) 'Caseins as source of bioactive peptides', *International Dairy Journal*, 15, 1–15.

Smyth, M. and FitzGerald, R. J. (1997) 'Characterisation of a new chromatography matrix for peptide molecular mass determination', *International Dairy Journal*, 7, 571-577.

Smyth, M. and FitzGerald, R. J. (1998) 'Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations', *International Dairy Journal*, 8, 819-827.

Srinivas, S. and Prakash, V. (2010) 'Bioactive peptides from bovine milk α-casein: Isolation, characterization and multifunctional properties', *International Journal of Peptide Research and Therapeutics*, 16, 7-15.

Stuchell, Y. M. and Krochta, J. M. (1995) 'Edible coating on frozen king salmon: Effect of whey protein isolate and acetylated monoglycerides on moisture loss and lipid oxidation', *Journal of Food Science*, 60, 28-31.

Suetsuna, K., Ukeda, H. and Ochi, H. (2000) 'Isolation and characterization of free radical scavenging activities peptides derived from casein', *Journal of Nutritional Biochemistry*, 11, 128-131.

Swaisgood, H. E. (2003) 'Chemistry of the caseins', in Fox, P. F. and McSweeney, P. L. H., eds., *Advanced Dairy Chemistry Volume 1: Proteins*, 3rd ed., New York, NY, USA: Kluwer Academic/Plenum Publishers, 139-201.

Teschemacher, H. and Brantl, V. (1994) 'Milk protein derived atypical opioid peptides and related compounds with opioid antagonist activity', in Brantl, V. and Teschemacher, H., eds., *β-casomorphins and Related Peptides: Recent developments*, Weinheim, GERMANY: VCM, 3-17.

Ueno, K., Mizuno, K. and Yamamoto, N. (2004) 'Purification and characterization of an endopeptidase that has an important role in the carboxyl terminal processing of antihypertensive peptides in *Lactobacillus helveticus* CM4', *Letters in Applied Microbiology*, 39, 313-318.

Vercruysse, L, Van Camp, J. and Smagghe, G. (2005) 'ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review', *Journal of Agricultural and Food Chemistry*, 53, 8106-8115.

Visser, S., Slangen, C. J., Lagerwerf, F. M., Van Dongen, W. D. and Haverkamp, J. (1995) 'Identification of a new genetic variant of bovine β-casein using reversed-phase high-performance liquid chromatography and mass spectrometric analysis', *Journal of Chromatography A*, 711, 141-150.

Wang, W. Y. and De Mejia, E. G. (2005) ‘A new frontier in soy bioactive peptides that may prevent age-related chronic diseases’, *Comprehensive Reviews in Food Science Food Safety* , 4, 63-78.

Wu, H.-C., Chen, H.-M. and Shiau, C.-Y. (2003) ‘Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*)’, *Food Research International*, 36, 949-957.

Xu, R. J. (1998) ‘Bioactive peptides in milk and their biological and health implications’, *Food Reviews International*, 14(1), 1-16.

Yamamoto, M., Akino, A. and Takano, T. (1994) ‘Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus CP790’, Journal of Dairy Science*, 77(4), 917-922.

Yamamoto, N., Akino, A. and Takano, T. (1999) ‘Purification and characterisation of an antihypertensive peptide from yoghurt-like produce fermented by *Lactobacillus helveticus* CPN4’, *Journal of Dairy Science*, 82, 1388-1393.

Zommara, M., Tougo, H., Sakanao, M. and Imaizumi, K. (1998) ‘Prevention of peroxidative stress in rats fed on a low vitamin E containing diet by supplementing with a fermented bovine milk whey preparation: Effect of lactic acid and beta-lactoglobulin on the antiperoxidative action’, *Bioscience, Biotechnology and Biochemistry*, 62, 710-717.

Zhu, L., Chen, J., Tang, X. and Xiong, Y. L. (2008) ‘Reducing, Radical Scavenging, and Chelation Properties of *in vitro* Digests of Alcalase-Treated Zein Hydrolysate’, *Journal of Agricultural and Food Chemistry*, 56(8), 2714-2721.

Zulueta, A., Esteve, M. J. and Frígola, A. (2009) ‘ORAC and TEAC assays comparison to measure the antioxidant capacity of food products’, *Food Chemistry*, 114, 310-316.

CHAPTER 5

Conclusions

This work aimed to characterize two plant coagulants, *Cynara cardunculus* L. and *Ficus carica* L., and improve their current knowledge.

Firstly, the presence of lipases was investigated. Both plant extracts possessed esterase and not lipase activity with major affinity for acetetate, propionate and butyrate esters. Moreover, the temperature and pH profiles showed that *Cynara cardunculus* and *Ficus carica* displayed optimal activity in a range temperature of 40-60°C and neutral-alkaline pHs (7.5-8.0). In particular, the zymographic profiles of *Ficus carica* esterase revealed several isoforms, differing in substrate specificity and electrophoretic mobility. Besides, it was highlighted that the clear supernatant (CS) and the gummy material (GM) of *Ficus carica*, separated by centrifugation, expressed a similar enzymatic activity.

In the second part of this thesis, the proteolytic activity of the two plant extracts was also characterized. *Cynara cardunculus* and *Ficus carica* expressed maximum activity at pH 5.0 and 7.0, respectively, and an optimal temperature at 60°C. To assess the contribution of each plant enzyme to the overall proteolytic specificity, the hydrolysis of bovine α_s and β-caseins was performed and examined by eletrophoretic gels and densitogram analysis. The extent of enzyme-mediated proteolysis was much higher for ficin than cardosin and depended on the type and specificity of proteinase, enzyme concentration, type of substrate as well as time of hydrolysis. Moreover, the study of fig proteinases by zymography revealed the presence of several isoforms, indicating a high level of polymorphism.

Finally, *Cynara cardunculus* L. and *Ficus carica* L. proteinases were investigated for their ability to produce antioxidant hydrolysates/peptides from bovine casein (CN). Since *Cynara cardunculus* protease reported low degree of hydrolysis (DH) on Na-CN, this plant extract was not considered as a good source of bioactive peptides. On the contrary, *Ficus carica* proteinase showed broad specificity and satisfactory DH both on Na-CN and β-CN. The ability of Na-CN and β-CN hydrolysates to have antioxidant activity appeared to be related to the degree of hydrolysis (DH) and the nature and composition of peptides, i.e., sequence and

size, produced at different stages of hydrolysis. In this regard, gel permeation HPLC showed that the antioxidant activity of β-CN hydrolysates depended on peptide material with MW < 500 Da, while UPLC/MS analysis seemed to confirm that the antioxidant activity of these peptides depended on the high levels of His, Leu, Pro residues and other hydrophobic amino acids.

In conclusion, main results of this thesis work suggest that the enzymatic characterization of these plant coagulants is useful for exploring their potentials applications in dairy industry. In this regard, further studies need to be carried out to assess the use of *Cynara cardunculus* and *Ficus carica* esterases and proteinases in cheese making and their contribution, respectively, on lipolysis and proteolysis, and development of flavour. Futhermore, health and biological benefits of antioxidant peptides originated by *Ficus carica* need to be investigated in depth. Finally, the use of these crude enzyme extracts in cheese technology might be perceived by customers as natural and healthy. On the other hand, dairy industries can promote the manufacture of artisanal and traditional cheeses in contrast to the “industrial” ones.