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Semisynthesis of carboxy-terminal fragments of thermolysin

VINCENZO DE FILIPPIS and ANGELO FONTANA

Department of Organic Chemistry, Biopolymer Research Centre of CNR, University of Padua, Padua, Italy

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Enzyme-catalyzed synthesis of two polypeptide fragments, one of which is obtained by chemical synthesis, in the presence of proteolytic enzymes and in aqueous organic solvents constitutes a convenient procedure for the synthesis of proteins and their analogs. This novel semisynthetic procedure was investigated for preparing *COOH*-terminal fragments of the metallo-protease thermolysin. Fragment 205–316, obtained by autolysis of the protein in the presence of EDTA, was first cleaved selectively with *Staphylococcus aureus* V8 protease at the level of the single Glu^{302} residue into fragments 205–302 and 303–316. Upon incubation for 2–5 days of fragment 205–302 with a 5-fold excess of peptide 303–316, prepared by solid phase synthesis, with V8-protease in 0.1 M ammonium acetate, pH 6.0, containing 50% glycerol as organic cosolvent, enzyme-catalyzed reformation of the peptide bond was achieved in yields up to ~90% (based on fragment 205–302). The same procedure was used to prepare also the thermolysin fragments 205–315 and 205–311 by enzymatic coupling of fragment 205–302 to peptide 303–315 or 303–311, these last prepared by proteolytic digestion of the synthetic peptide 303–316. This procedure of semisynthesis opens up an approach for the site-directed modification of the tetrahelical *COOH*-terminal fragment 205–316 of thermolysin at the level of its helical segment encompassing residues 301–312 in the native, intact protein. Such analogs will be useful for examining structure-folding-stability relationships in this folded fragment possessing domain-like characteristics.

Key words: peptide synthesis; protein domains; peptide synthesis; semisynthesis; thermolysin; V8-protease

The ability of proteolytic enzymes to catalyze peptide bond synthesis has been investigated over the last years with the aim of developing useful methods to produce biologically active polypeptides (1–5). Proteases can form peptide bonds either by stepwise addition of amino acid residues to the *C*-terminus of polypeptide substrates by the use of the exopeptidase carboxypeptidase Y (6) or by fragment condensation of two polypeptide fragments by the use of endopeptidases such as trypsin, chymotrypsin, subtilisin and thermolysin (7–9). While the stepwise elongation of a polypeptide at its *C*-terminus by carboxypeptidase Y takes advantage of both the esterase and amidase activity of the enzyme (4, 6), fragment coupling can be achieved by influencing the equilibrium concentration of products and kinetic parameters of the enzymatic reaction (1–5, 7–9). In general, the synthetic action of endopeptidases is explored under conditions of thermodynamic control of the reaction and by the use of a rather high concentration of the amino component in the coupling reaction. A general approach to enhance synthesis over hydrolysis of peptide bonds by the use of endopeptidase consists of decreasing water activity in the reaction medium by adding water-miscible organic solvents such as glycerol, n-propanol, dimethylformamide, acetonitrile or dimethylsulfoxide (7–9). The use of a "molecular trap" that strongly binds to the newly enzyme-synthesized polypeptide was also shown to shift the equilibrium of the enzyme-catalyzed reaction (10, 11).

In recent years great interest was devoted to the enzymatic "semisynthesis" of protein molecules, defined as the rebuilding of a polypeptide chain from two components, one of which is obtained by chemical

Abbreviations used: RP-HPLC, reverse-phase high performance liquid chromatography; EDTA, ethylene-diaminotetraacetic acid; Gdn·HCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

Enzymes: Protease from *Staphylococcus aureus* V8 (EC 3.4.17.19); thermolysin (EC 3.4.24.4); carboxypeptidase B (EC 3.4.21.2).

synthesis (12–20). The advantages of this procedure lie in the possibility of obtaining a rather large polypeptide chain with minimum use of protecting groups and chemical manipulations and without the undesirable side products usually generated in the chemical (solid-phase) synthesis of rather long peptides (21). General problems of the enzymatic "semisynthesis" of proteins consist in that large polypeptide fragments usually contain several potential sites of undesirable cleavage by the endopeptidase used and may possess a three-dimensional structure rendering difficult the coupling reaction by steric constraints. However, despite these limitations, the "semisynthetic" approach of proteins in special cases can be used with great success and flexibility, as already extensively documented (15–20, 22), complementing current methods of protein engineering of protein molecules by the use of genetic methods (23, 24) (cf. ref. 15, for an excellent discussion on alternatives to recombinant methods in order to achieve protein engineering).

In this paper we report the use of the semisynthetic approach in preparing rather long polypeptide fragments of the carboxy-terminal domain of the metalloendoprotease thermolysin (25, 26). The procedure here described takes advantage of the availability of fragment 205–316 of thermolysin, obtained in high yields by specific autolysis of the 316-residue chain of the metallo-protease in the presence of EDTA (27). A unique feature of this fragment is that its polypeptide chain of 112 amino acid residues, lacking disulfide or thiol groups, contains a single glutamic acid residue in position 302 of the chain (see Fig. 1). This peculiar structural feature was exploited for specifically cleaving fragment 205–316 at Glu^{302} with *Staphylococcus aureus* V8 protease (28, 29) and for preparing in homogeneous form and high yields fragment 205–302. This last fragment was coupled in aqueous glycerol in the presence of V8-protease to peptide 303–316 prepared by solid-phase-methods (21), as well as to peptides 303–315 and 303–311 obtained from the synthetic peptide 303–316 by proteolytic digestion. Correspondingly, fragments 205–316, 205–315, and 205–311 have been prepared and isolated in homogeneous form in high yields. The semisynthesis procedure here described opens an opportunity to study conformational and stability properties of "mutants" of carboxy-terminal fragments of thermolysin, previously shown to maintain in aqueous solution a folded and stable conformation of native-like characteristics and thus to possess "protein domain" properties (30–34).

MATERIALS AND METHODS

Materials

Thermolysin from *Bacillus thermoproteolyticus* (Rokko) was obtained from Sigma Chem. Co. (St. Louis, MO, USA) as a lyophilized product containing about 10% autolytic products and 30% calcium and sodium acetate. The protease from *Staphylococcus aureas* V8 was purchased from Miles (Elkhart, IN, USA) and carboxypeptidase B from Boehringer (Mannheim, FRG).

Trifluoroacetic acid (TFA), ultrapure guanidine hydrochloride (Gdn·HCl), EDTA, formic acid and Tris were reagent grade materials obtained from Fluka (Basle, Switzerland). The materials used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Richmond, CA, USA). Deionized water obtained from a Milli-Q system (Millipore Inc., Milford, MA, USA) was used for all solutions. All other chemical compounds were of analytical grade and were obtained from C. Erba (Milan, Italy) or Merck (Darmstadt, FRG).

Methods

High performance liquid chromatography. Separations of peptide mixtures were obtained using HPLC equipment obtained from LKB (Bromma, Sweden) and consisted of two model 2150 pumps, a gradient controller model 2152, an Uvicord SD model 2158 ultraviolet detector and a model 2210 two-pen recorder. Samples were injected using a Rheodyne model 7125 septumless injection valve equipped with injection loops of different size. The column eluent was monitored at 226 nm and fractions were collected manually in 1.5 mL polypropylene tubes (Eppendorf). The following columns were used for the purification of peptide mixtures. (a) Aquapore (30-nm pore size, 7-μm particle diameter, octyl- or butyl-silica packed into a stainless steel column, 4.6 × 100 mm) (Brownlee Laboratories, Santa Clara, CA, USA). (b) μBondapak C_{18} (3.9 × 150 mm or a semipreparative column

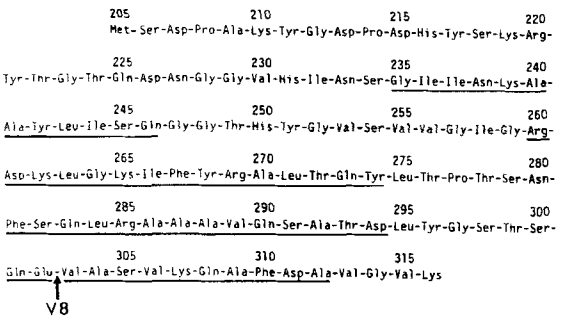

FIGURE 1

Amino acid sequence of the carboxy-terminal fragment 205–316 of thermolysin (25).

The single site of cleavage by staphylococcal V8-protease at Glu^{302} is indicated by an arrow. The four helical segments in the crystallographically determined structure of thermolysin (26) are underscored.

7.8 × 300 mm, 12.5–45 nm pore size, 10-μm particle diameter, octadecyl-silica).

Polyacrylamide gel electrophoresis in the presence of SDS was carried out using a vertical slab gel apparatus according to the procedure of Laemmli (35). An exponential acrylamide gradient from 15 to 24% along the direction of migration was used. The gel thickness was 1 mm. As molecular weight markers were used cyanogen bromide fragments of both thermolysin (36) and horse heart cytochrome C (37) previously obtained in our laboratory.

Thin-layer chromatography (TLC) was performed on cellulose plates (Merck) in n-butanol:acetic acid: water (3:1:1, by vol.). Location of peptides in the plates was revealed with ninhydrin.

Amino acid analyses were performed using the Millipore-Waters Pico-Tag work-station and the Pico-Tag column (3.9 × 150 mm) connected to a Perkin-Elmer model LC-410 liquid chromatograph equipped with a variable detector model LC-95 and with a data processor system model 7700 utilizing the computer program Chrom-3. Lyophilized samples of peptides (50–1000 pmol), contained in heat-treated borosilicate tubes (4 × 50 mm), were acid-hydrolyzed on the Pico-Tag work-station for 60 min at 150° using 200 μL of 6 N HCl containing 0.1% phenol. Some amino acid analyses were also performed on a C. Erba (Milan, Italy) automatic amino acid analyzer, model 3A29, after 22-h hydrolysis at 110° in 6 N HCl in evacuated, sealed tubes.

Peptide concentrations were determined by quantitative amino acid analysis of acid hydrolyzates of aliquots taken from peptide solutions. Alternatively, with thermolysin fragments containing tyrosine, the concentration was determined by absorption determination at 280 mm, assuming a molar extinction coefficient by 1280 $M^{-1} \cdot cm^{-1}$ for tyrosine (38). It was found that the two methods of analysis corresponded favorably ($\pm$ 3%).

Preparation of fragments 205–316 and 205–302

Fragment 205–316. This fragment was prepared taking advantage of the almost quantitative autolytic cleavage of thermolysin at peptide bonds 196–197 and 204–205 in the presence of EDTA, leading to a "nicked" protein constituted by fragments 1–196, 197–204, and 205–316 associated in a stable complex (27). To a solution of commercial thermolysin (as obtained from Sigma, see Materials) (100 mg) in 100 mL of 10 mM Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl, EDTA (0.4 M in Tris buffer) was added under stirring to a final concentration of 50 mM. The solution was gently stirred at room temperature for 2 h, filtered from some undissolved material through a 0.45 μm Millipore filter and then added dropwise under vigorous stirring to 25 mL of a 50% aqueous formic acid solution. The suspension thus obtained was kept overnight at 4° and the precipitate was separated by centrifugation. The precipitate was suspended twice in 10 mL of 1% aqueous acetic acid and again centrifuged. All the supernatants were combined, concentrated *in vacuo* to about 40 mL, dialyzed against 1% aqueous acetic acid for two days and then the protein material was recovered by lyophilization. The overall yield of homogeneous fragment was ~30% (based on protein content of the thermolysin sample employed). The identity and purity of this fragment was established by electrophoresis, HPLC, amino acid analysis, and amino-terminal sequencing (see Results and also ref. 27).

Fragment 205–302. A solution of fragment 205–316 (1.5 mg; 125 nmol) in 800 μL of 0.1 M ammonium bicarbonate, pH 7.8, containing 1 mM EDTA and 0.2% SDS was heated at 70° for 10 min. The reaction mixture was then cooled at 20° and 35 μL of a solution of V8-protease (2 mg/mL in bicarbonate buffer) were added (peptide:protease ratio of 20:1, by weight). The sample was kept at room temperature for 24 h and aliquots were removed at intervals and analyzed by RP-HPLC. Digestion was terminated by adding 20 μL of 50% aqueous TFA. To the sample were added 40 μL of 7 M Gdn·HCl and the precipitate was separated by centrifugation at 15 000 rpm for 5 min. This procedure was found useful for effectively removing SDS from the sample solution (see also Results). The clarified Gdn·HCl solution of the reaction mixture was then fractionated by RP-HPLC.

Preparation of peptides 303–316, 303–315, and 303–311

Peptide 303–316. This 14-amino acid peptide, corresponding to residues 303–316 of thermolysin (see Fig. 1), was synthesized on a semi-automatic solid-phase synthesizer NPS-4000 at Neosystem Laboratories (Strasbourg, France) following essentially the procedure of Merrifield and using benzyl-type protecting groups (21). The crude peptide, as obtained after HF-cleavage from the resin, was purified by RP-HPLC as described under Results and its purity checked by TLC and amino acid analysis.

Peptide 303–315. In order to remove the *COOH*-terminal Lys^{316} from the synthetic peptide 303–316 purified to homogeneity (see above), carboxypeptidase B digestion of the peptide (1.5 mg; 1.1 μmol) was carried out for 30 min at 20° in 1.1 mL of 0.1 M

ammonium bicarbonate buffer, pH 7.8, at a peptide to enzyme ratio of 10:1, by weight. Following digestion, the sample was acidified by the addition of 100 μL of 50% aqueous TFA and then dried on a Speed-Vac concentrator. Homogeneous peptide 303–315 was isolated from the residue by RP-HPLC.

Peptide 303–311. This peptide was obtained by cleaving the synthetic peptide 303–316, at Asp^{311} with *Staphylococcus aureus* V8 protease, taking advantage of the fact that this protease shows a buffer- and pH-dependent specificity towards peptide bonds involving Glu or Asp residues, the former being cleaved preferentially in ammonium bicarbonate buffer, pH 7.8, or ammonium acetate buffer, pH 4.0, and both in sodium or potassium phosphate buffer, pH 7.8 (29). The peptide 303–316 (2 mg; 1.4 μmol) was dissolved in 1.9 mL of 0.1 M sodium phosphate buffer, pH 7.8, and incubated with V8-protease at a peptide:enzyme ratio of 20:1, by weight, for 18 h at 37°. Proteolysis was terminated by adding 100 μL of 50% aqueous TFA and then the sample was dried on a Speed-Vac concentrator. The desired peptide 303–311 was isolated to homogeneity from the reaction mixture by RP-HPLC.

Semisynthesis of thermolysin fragments

Fragment 205–316. To a solution of the thermolysin fragment 205–302 (0.55 mg; 52 nmol) and the synthetic peptide 303–316 (0.4 mg; 281 nmol) in 100 μL of 0.1 M ammonium acetate buffer, pH 6.0, containing 50% glycerol, 3 μL of a solution of V8-protease (2 mg/mL in acetate buffer) were added. The enzymatic reaction was allowed to proceed at room temperature (20–22°) for 5 days. At time intervals, aliquots were removed from the reaction mixture and analyzed by RP-HPLC. Additional experiments of enzymatic fragment coupling were performed under different experimental conditions and in particular varying the temperature of incubation, as well as nature and percentage of the organic cosolvent (see Results).

Fragment 205–315. Fragment 205–302 (0.55 mg; 52 nmol) and peptide 303–315 (0.37 mg; 280 nmol) in 100 μL of 0.1 M ammonium acetate, pH 6.0, containing glycerol to a final concentration of 50%, were incubated with V8-protease (6 μg) for 4 days at room temperature. The time-course of the semisynthesis reaction was monitored by HPLC analysis.

Fragment 205–311. Fragment 205–302 (0.28 mg; 27 nmol) and peptide 303–311 (0.14 mg; 145 nmol) were incubated in the presence of V8-protease (2.8 μg) for 4 days at room temperature in 50 μL of 0.1 M ammonium acetate, pH 6.0, containing glycerol to a final concentration of 50%.

RESULTS

Preparation of fragments and peptides

Fragments 205–316 and 205–302. In a previous study (27) it was shown that thermolysin can be induced to autolyze almost quantitatively when incubated at pH 7.2 in the presence of EDTA at the level of peptide bonds Gly^{196}-Ile^{197} and Ser^{204}-Met^{205}. Interestingly, the resulting three fragments 1–196, 197–204, and 205–316 remain associated in a stable, folded complex. The autolytic reaction of thermolysin was employed herewith to prepare in homogeneous form and 30% yields the carboxy-terminal fragment 205–316. It is worth noting that the fragment can be prepared directly from a commercially available sample of thermolysin treated with EDTA and by using a simple procedure which does not necessarily require column chromatographic steps (see Methods). The homogeneity and identity of the isolated fragment 205–316 was established by SDS-PAGE, HPLC, amino acid analysis, and partial sequencing (see Table 1 and also ref. 27). Initial attempts to cleave fragment 205–316 at the level of the single Glu^{302} (see Fig. 1) by *Staphylococcus aureus* V8 protease in aqueous buffer, pH 7.8, were not successful, since the fragment was found quite resistant to proteolysis even after prolonged incubation with V8-protease at temperatures between 20° and 37°, as demonstrated by RP-HPLC analysis of an aliquot of the proteolytic mixture (Fig. 2A). This was interpreted as an indication that the folded conformation of the fragment renders quite inaccessible to the active site of the V8-protease the peptide segment near Glu^{302}.

Efficient cleavage was achieved when fragment 205–316 was first denatured upon heating at 70° for 10 min in aqueous buffer, pH 7.8, containing 0.2% SDS and then incubated in the same buffer mixture for 24 h with V8-protease at a fragment:enzyme ratio of 20:1 (by weight). The RP-HPLC chromatogram of an aliquot of the proteolytic mixture (Fig. 2B) indicates that the digestion of fragment is over 90% under the given experimental conditions, since trace amounts of the substrate fragment 205–316 are present in the chromatogram and two new major peaks of peptide material are seen. In order to obtain successful and reproducible separation of the V8-protease proteolytic mixture it was found of critical importance to add to the sample, before RP-HPLC analysis, a Gdn·HCl solution in order to remove the detergent SDS by precipitation (see Methods). Additional experiments (not shown) indicated that the proteolysis of fragment 205–316 mediated by V8-protease can be induced also by adding to the proteolytic mixture other protein denaturants such as Gdn·HCl, but the yields of cleavage were lower than those obtained in the presence of SDS. Fractions corresponding to the peaks of the chromatogram shown in Fig. 2B were combined and

TABLE 1

Amino acid composition of thermolysin fragments, synthetic peptides and semisynthetic fragments[a]

Amino acid	Fragments and synthetic peptides					Semisynthetic fragments		
	205–316	205–302	303–316	303–315	303–311	205–316	205–315	205–311
Aspartic acid	10.2 (11)	8.9 (10)	0.7 (1)	1.0 (1)	1.2 (1)	9.9 (11)	9.5 (11)	9.5 (11)
Glutamic acid	7.8 (8)	7.1 (7)	0.9 (1)	1.0 (1)	1.0 (1)	7.9 (8)	8.2 (8)	8.4 (8)
Serine	10.8 (11)	9.9 (10)	0.9 (1)	0.8 (1)	0.9 (1)	10.6 (11)	10.5 (11)	10.6 (11)
Glycine	13.1 (13)	12.2 (12)	1.1 (1)	1.1 (1)	– (0)	12.9 (13)	13.2 (13)	12.8 (12)
Histidine	2.7 (3)	2.8 (3)	– (0)	– (0)	– (0)	2.5 (3)	2.8 (3)	2.4 (3)
Arginine	4.2 (4)	4.5 (4)	– (0)	– (0)	– (0)	4.1 (4)	4.2 (4)	4.6 (4)
Threonine	7.6 (8)	7.8 (8)	– (0)	– (0)	– (0)	7.8 (8)	7.9 (8)	7.5 (8)
Alanine	11.2 (11)	7.7 (8)	3.1 (3)	3.0 (3)	2.0 (2)	10.9 (11)	11.0 (11)	9.9 (10)
Proline	3.0 (3)	3.0 (3)	– (0)	– (0)	– (0)	3.1 (3)	2.8 (3)	3.2 (3)
Tyrosine	7.8 (8)	7.9 (8)	– (0)	– (0)	– (0)	7.6 (8)	7.8 (8)	7.6 (8)
Valine	9.0 (9)	4.9 (5)	4.1 (4)	3.9 (4)	1.9 (2)	9.0 (9)	8.9 (9)	7.0 (7)
Methionine	0.9 (1)	0.7 (1)	– (0)	– (0)	– (0)	1.1 (1)	0.8 (1)	0.9 (1)
Isoleucine	5.6 (6)	5.5 (6)	– (0)	– (0)	– (0)	5.8 (6)	5.6 (6)	5.7 (6)
Leucine	6.0 (6)	5.6 (6)	– (0)	– (0)	– (0)	5.7 (6)	5.8 (6)	6.4 (6)
Phenylalanine	3.0 (3)	2.0 (2)	1.0 (1)	1.0 (1)	1.0 (1)	3.0 (3)	3.0 (3)	3.0 (3)
Lysine	6.8 (7)	4.9 (5)	1.9 (2)	1.0 (1)	1.0 (1)	6.9 (7)	5.8 (6)	6.0 (6)

[a] Amino acid compositions are reported as amino acid residues per molecule. Expected values are given in parentheses and were calculated on the basis of the amino acid sequence of thermolysin. The figures given are average values obtained usually from at least two separate amino acid analyses. The amino acids are listed in the order they elute from the Pico-Tag column (see Methods). The thermolysin fragments, synthetic peptides, and semisynthetic fragments were prepared as described in the text.

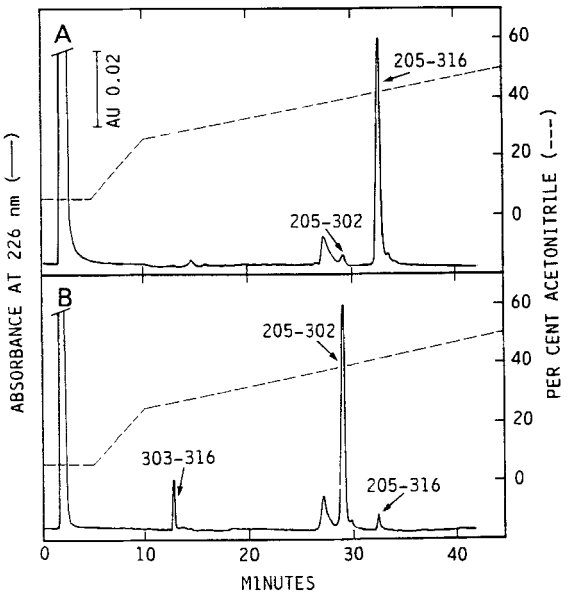

FIGURE 2

RP-HPLC analysis of the V8-protease digest of fragment 205–316. An aliquot of the proteolytic mixture (0.1 M ammonium bicarbonate, pH 7.8) was applied to an Aquapore Butyl column (4.6 × 100 mm) and elution was carried out at room temperature at a flow rate of 0.8 mL/min using a gradient of acetonitrile in 0.05% (by vol.) aqueous TFA, as shown by the dashed line. *A.* V8-digest, 24-h, 20°. *B.* V8-digest in the presence of SDS 0.2%, 24-h, 20°. Numbers near the chromatographic peaks refer to the identity of the peptide material.

the peptide material recovered by lyophilization. The fragments thus isolated were shown to be homogeneous by RP-HPLC employing different (C_4-, C_8- and C_{18}-silica) columns and by SDS-PAGE analysis (not shown). The identity of the fragments was established by amino acid analysis (Table 1) and partial sequencing and comparing these data with the known amino acid sequence of thermolysin (25). The peptide material eluting ahead of fragment 205–302 (see Fig. 2) was heterogeneous and contained degradation products of V8-protease.

Peptides 303–316, 303–315, and 303–311. Even if the peptide 303–316 could be prepared and isolated to homogeneity from fragment 205–316 by digestion with V8-protease (see above), it was considered advisable to prepare relatively large quantities of this peptide by solid-phase methods for the purposes of present and future studies.

The peptide 303–316 was assembled on a Merrifield-type resin and after HF-cleavage from the resin 0.5 g of crude material was obtained (21). An aliquot of this crude peptide was purified in a single step to homogeneity by RP-HPLC using a μBondapak C_{18} column eluted with a water-TFA-acetonitrile system (see Fig. 3A). The purified tetradecapeptide thus isolated was homogeneous on the basis of quantitative amino acid analysis of an acid hydrolyzate (Table 1), TLC and fingerprinting analysis (see below). In addition, a mixture of synthetic and

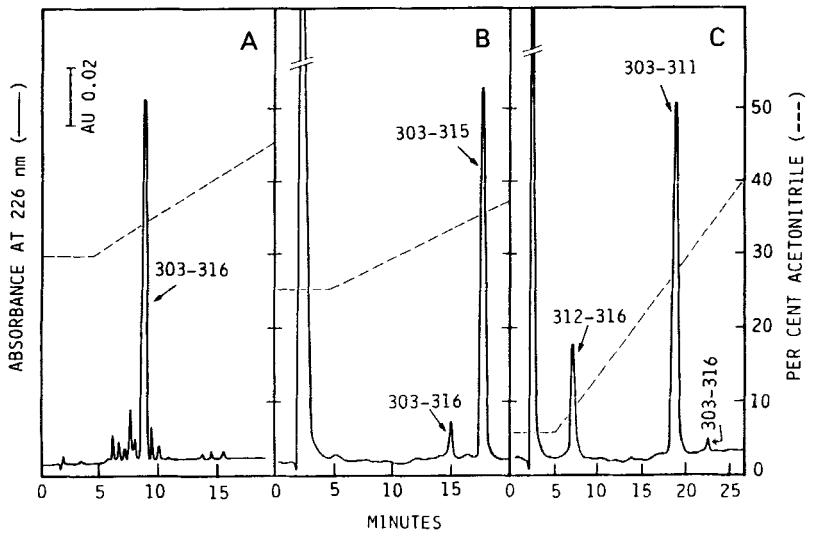

FIGURE 3

Purification by RP-HPLC of the synthetic peptide 303–316 of thermolysin (A) and its proteolytic fragments produced by carboxypeptidase B (B) and V8-protease (C) digestion.

A μBondapak C_{18} column (3.9 × 150 mm) was employed and eluted at room temperature at a flow rate of 0.5 mL/min, utilizing a gradient of acetonitrile in 0.05% (by vol.) aqueous TFA, as shown by the dashed line. Numbers near the chromatographic peaks refer to the peptides identified (see Results).

authentic peptide 303–316 prepared by V8-protease digestion of fragment 205–316 (see above) eluted as a single peak from the RP-HPLC column (not shown).

Proteolytic digestion of the homogeneous synthetic peptide 303–316 was employed to prepare two additional peptides to be used for the subsequent semisynthesis experiments. Carboxypeptidase B digestion of the peptide at pH 7.8 was used to remove quantitatively its *COOH*-terminal Lys^{316}. Incubation of the peptide in sodium phosphate buffer, pH 7.8, in the presence of V8-protease for 18 h leads to efficient splitting of the peptide bond involving Asp^{311}. This results from the fact that V8-protease can cleave also at Asp under special buffer conditions (see ref. 29). From these two proteolytic mixtures the truncated peptides 303–315 and 303–311 were isolated by RP-HPLC on a μBondapak C_{18} column (Fig. 3B and C). Homogeneity of these peptides was established by TLC and amino acid analysis (Table 1).

Enzyme-catalyzed fragment condensations

The V8-protease synthesis of the peptide bond Glu^{302}-Val^{303} between fragment 205–302 and synthetic peptide 303–316 was carried out in 0.1 M ammonium acetate buffer, pH 6.0, in the presence of 50% glycerol. These experimental conditions were analogous to those previously employed in a study of semisynthesis of α-chain of hemoglobin S, with the difference that in the present study glycerol was used as organic cosolvent instead of n-propanol (17). The time-course of the enzyme-catalyzed synthesis was followed by RP-HPLC analysis of aliquots taken from the reaction mixture (Figs. 4 and 5). Before addition of V8-protease, the sample contained only fragment 205–302 and synthetic peptide 303–316, this last being added in about five-fold (molar ratio) excess over the other component (Fig. 4A). Upon addition of V8-protease,

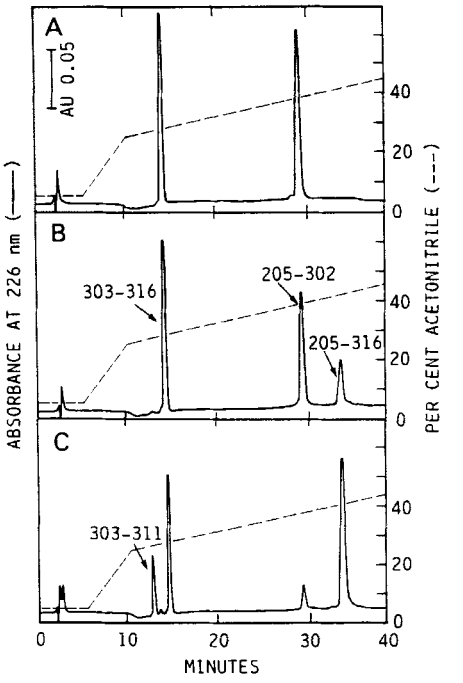

FIGURE 4

Separation of the reaction mixture of the V8-protease mediated coupling of fragment 205–302 to the synthetic peptide 303–316 by RP-HPLC.

The analysis was carried out utilizing an Aquapore RP-300 C_8 column (4.6 × 100 mm) eluted at room temperature at a flow rate of 0.8 mL/min utilizing a gradient of acetonitrile in 0.05% (by vol.) aqueous TFA, as shown by the dashed line. The reaction was carried out as described under Methods and aliquots of the reaction mixture were analyzed before (A) and after 3-h (B) and 90-h (C) addition of V8-protease. Numbers near the chromatographic peaks refer to the identity of the peptide material eluted from the column.

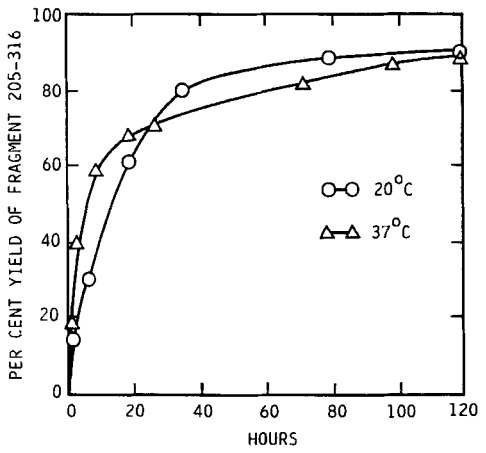

FIGURE 5

Time-course of the yields of V8-protease mediated semisynthesis of fragment 205—316 from fragment 205–302 and the synthetic peptide 303–316.

The coupling reaction was carried out as described under Methods at 20° (○) and 37° (△). Aliquots were removed at intervals from the mixture and analyzed by RP-HPLC using the conditions described in the legend to Fig. 4. Yields of resynthesis were calculated by a computer-assisted quantitative analysis of the chromatograms.

a new component was generated eluting from the HPLC column after fragment 205–302, whereas the amount of this last fragment was significantly reduced (see Fig. 4B and C). Considering that the new component eluted at the same position as authentic fragment 205–316 and that its amino acid analysis was in good agreement with the expected composition of fragment 205–316 (Table 1), it was concluded that very effective enzymatic semisynthesis had occurred. In addition, tryptic peptide mapping by RP-HPLC of the semisynthetic compound was identical to that given by authentic fragment 205–316 (not shown). An additional peak is seen in the RP-HPLC chromatogram of the reaction mixture after 90-h reaction, preceding the peak of peptide 303–316 (Fig. 4C). The peptide material of this peak gave an amino acid analysis consistent with sequence 303–311, indicating that, under the given experimental conditions of the semisynthesis reaction, V8-protease can cleave the synthetic peptide 303–316 at the level of the Asp^{311}-Ala^{312} peptide bond.

Figure 5 shows a kinetic analysis of the yields of semisynthesis of fragment 205–316 performed at 20° and 37°. From these data, it can be seen that the yields of enzymatic peptide bond formation can be as high as ~90%, when the reaction is performed at pH 6.0 in the presence of 50% glycerol using a five-fold molar excess of synthetic peptide 303–316 and a 90-h reaction time. As shown earlier in other studies of semisynthesis, organic cosolvents such as n-propanol, ethyleneglycol, acetonitrile or dimethylsulfoxide can be employed (7–10, 17), but in the present thermolysin fragment system the yields of synthesis were lower in respect to those observed in the presence of glycerol.

The semisynthetic procedure was also employed to obtain fragments 205–315 and 205–311 by enzymatic condensation of fragment 205–302 with 5 equivalents of peptide 303–315 or 303–311, these last obtained by proteolytic digestion of the synthetic fragment 303–316 (see above). The experimental conditions were essentially those employed for the semisynthesis of fragment 205–316. Fig. 6 shows typical RP-HPLC chromatograms of corresponding reaction mixtures. The yields of fragments 205–315 and 205–311 determined by RP-HPLC (see above) were ~90% and 48% (based on fragment 205–302), respectively. The amino acid analyses of the fragments thus produced after acid hydrolysis were in agreement with those calculated for thermolysin fragments 205–315 and 205–311 (see Table 1).

DISCUSSION

The results presented here show that *Staphylococcus aureus* V8 protease is able to catalyze in the presence of organic solvents the synthesis of peptide bond Glu^{302}-Val^{303} when fragment 205–302 of thermolysin is mixed with the shorter, synthetic peptide 303–316. A striking feature of the semisynthesis here described is

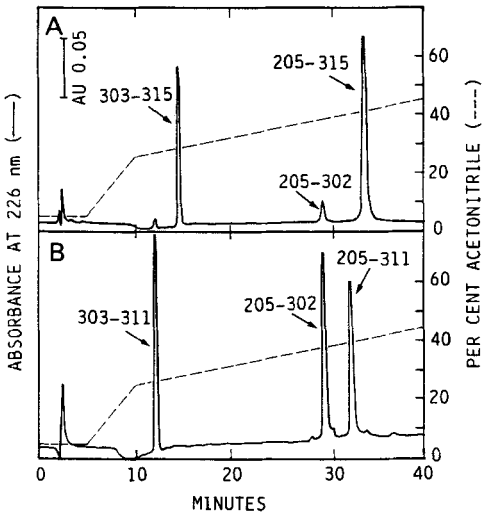

FIGURE 6

RP-HPLC analysis of the reaction mixture of the V8-protease mediated coupling of fragment 205–302 with peptide 303–315 (A) or peptide 303–311 (B).

The analysis was carried out utilizing an Aquapore RP-300 C_8 column (4.6 × 100 mm) eluted with a gradient of acetonitrile in 0.05% (by vol.) aqueous TFA, as shown by the dashed line, at a flow rate of 0.8 mL/min. The semisynthesis reaction was carried out as described under Methods and an aliquot was analyzed after 90 h (A) and 96 h (B).

that it occurs cleanly and in high yields (up to 90%), when the amino component in the coupling reaction is employed in excess (5 equiv.). The procedure allows preparation of a rather long polypeptide chain of 112 amino acids, the size of a typical globular protein (e.g., ribonuclease, cytochrome C, etc.). Indeed, fragment 205–316 shows protein domain properties, being folded into a stable and native-like three-dimensional structure independently from the rest of the polypeptide chain of thermolysin. It was shown (unpublished experiments) that the conformational and stability properties of this fragment, examined by circular dichroism measurements, are identical to those already reported for the cyanogen bromide fragment 206–316 previously extensively studied in our laboratory (30–34), indicating that apparently the extra methionine residue at the amino terminus does not influence the fragment folding.

The semisynthesis procedure here described allows the preparation of analogs of the *COOH*-terminal globular domain of thermolysin. Thus, it will be possible to obtain sequence permutations at the level of one of the four helices of the thermolysin domain (see Fig. 1) and thereby to study the effects of amino acid side chain groups in both intra- and inter-helical interactions and packing. Likely, this newly developed system of analogs of thermolysin fragments will be a useful model to address the problem of helix stability and interactions, which is central in modern protein folding studies and under active investigation using both synthetic mutants of short peptides (40–43) and mutants of protein molecules prepared by site-directed mutagenesis techniques (44–47).

In other studies of protein semisynthesis it has been demonstrated that the enzymatic peptide bond formation is effective only if the two fragments interact to form a non-covalent complex of native-like structural properties, as seen in semisynthesis experiments of ribonuclease (7, 8, 10), nuclease (14), cytochrome C (48), α-chain of hemoglobin S (17). Complex formation appears to play a dominant role in the efficient resynthesis of the peptide bond by favoring the stereospecific orientation of the α-carboxyl and α-amino groups that are to form the peptide bond, so that these groups are close to one another as well as accessible to the protease. On this basis, the highly efficient semisynthesis of fragments 205–316, 205–315, and 205–311 here described prompts the proposal of a mechanism of fragment complexation. However, direct evidence of this has not been obtained yet and must await the outcome of subsequent studies. At any rate, the lower yields of enzymatic coupling to fragment 205–302 of a nonapeptide 303–311 (48%) in respect to the tetradecapeptide 303–316 (90%) seems to indicate that a proper chain length and thereby suitable interactions are required for an efficient semisynthesis.

The high yields of semisynthesis here observed can be a result of the fact that the end products of the enzymatic reaction are highly resistant to the proteolytic action of V8-protease, so that they are able to accumulate in the semisynthetic reaction mixture. This proposal appears to be substantiated by the observation that the native fragment 205–316 is quite resistant to the proteolytic action of V8-protease and that is being digested only after SDS- or Gdn·HCl-denaturation (see Results). Indeed, since the Glu^{302}-Val^{303} peptide bond is embedded in a helix segment in the intact thermolysin molecule (26) (and thus likely also in the fragment 205–316 of nativelike conformation), this bond is expected to be resistant to proteolytic fission under native conditions, considering that limited proteolysis of globular proteins occurs at exposed and flexible loops (cf. refs. 49 and 50, for a discussion on the mechanism of limited proteolysis of globular proteins).

In summary, this study opens up an opportunity to prepare a set of analogs of *COOH*-terminal fragments of thermolysin to be used for addressing the important problem of location, size, folding and stability of domains and subdomains in globular proteins (51–55). The novel procedure here described provides greater experimental latitude to the detailed conformational and stability studies on fragments of thermolysin prepared previously in our laboratory by chemical (30–33) as well as enzymatic (34) fragmentation of the protein. Analogs of fragment 205–316 with amino acid exchanges expected to alter the stability of the *COOH*-terminal helix, and thus the overall stability of the globular fragment, as well as analogs containing a tryptophan residue in position 310 of the polypeptide chain to be used as a fluorescent marker, are being synthesized and characterized. The results of these studies will be reported in subsequent publications.

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REFERENCES

1. Fruton, J. (1982) *Adv. Enzymol.* **53,** 239–306
2. Chaiken, I.M., Komoriya, A., Ohno, M. & Widmer, F. (1982) *Appl. Biochem. Biotechnol.* **7,** 385–399
3. Jakubke, H.-D., Kuhl, P. & Konnecke, A. (1985) *Angew. Chem. Int. Ed. Engl.* **24,** 85–93
4. Morihara, K. (1987) *Trends Biotechnol.* **5,** 164–170
5. Kullman, W. (1987) *Enzymatic Peptide Synthesis,* CRC Press, Boca Raton, FL
6. Breddam, K., Widmer, F. & Johansen, J.T. (1981) *Carlsberg Res. Commun.* **46,** 121–128

7. Homandberg, G.A., Mattis, J.A. & Laskowski, M., Jr. (1978) *Biochemistry* **17,** 5220–5227
8. Homandberg, G.A. & Laskowski, M., Jr. (1979) *Biochemistry* **18,** 586–592
9. Komoriya, A., Homandberg, G.A. & Chaiken, I.M. (1980) *Int. J. Peptide Protein Res.* **16,** 433–439
10. Homandberg, G.A., Komoriya, A. & Chaiken, I.M. (1982) *Biochemistry* **21,** 3385–3389
11. Nyberg, F. (1988) *J. Mol. Recognition* **1,** 59–62
12. Offord, R.E. (1969) *Nature* **221,** 37–47
13. Sheppard, R.C. (1980) in *The Peptides* (Gross, E. & Meienhofer, J., eds.), Vol. 2, pp. 441–484, Academic Press, New York
14. Chaiken, I.M. (1981) *CRC Crit. Rev. Biochem.* **11,** 255–301
15. Offord, R.E. (1987) *Protein Eng.* **1,** 351–357
16. Ten Kortenaar, P.B., Adams, P.J. & Tesser, G.I. (1985) *Proc. Natl. Acad. Sci. US* **82,** 8279–8283
17. Seetharam, R. & Acharya, A.S. (1986) *J. Cell. Biochem.* **30,** 87–99
18. Rose, K., Herrero, C., Proudfoot, A.E.J., Offord, R.E. & Wallace, C.J.A. (1988) *Biochem. J.* **249,** 83–88
19. Simmerman, H.K.B., Wang, C.C., Horowitz, E.M., Berzofsky, J.A. & Gurd, F.R.N. (1982) *Proc. Natl. Acad. Sci. US* **79,** 7739–7743
20. Jain, M.K., Malival, B.P., De Haas, G.H. & Slotboom, A.J. (1986) *Biochim. Biophys. Acta* **860,** 448–461
21. Van Regenmortel, M.H.V., Briand, J.P., Muller, S. & Plaue, S. (1988) in *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 19, pp. 1–227, Elsevier, Amsterdam
22. Offord, R.E. & Di Bello, C. (eds.) (1978) *Semisynthetic Peptides and Proteins*, Academic Press, London
23. Leatherbarrow, R.J. & Fersht, A. (1986) *Protein Eng.* **1,** 7–16
24. Oxender, D.L. & Fox, C.F. (eds.) (1987) *Protein Engineering*, Alan R. Liss, New York
25. Titani, K., Hermodson, M.A., Ericsson, L.H., Walsh, K.A. & Neurath, H. (1972) *Biochemistry* **11,** 2427–2435
26. Holmes, M.A. & Matthews, B.W. (1982) *J. Mol. Biol.* **160,** 623–639
27. Fassina, G., Vita, C., Dalzoppo, D., Zamai, M., Zambonin, M. & Fontana, A. (1986) *European J. Biochem.* **156,** 221–228
28. Drapeau, G.R. & Houmard, J. (1972) *Proc. Natl. Acad. Sci. US* **69,** 3506–3509
29. Drapeau, G.R. (1977) *Methods Enzymol.* **47,** 189–191
30. Fontana, A., Vita, C. & Chaiken, I.M. (1983) *Biopolymers* **22,** 69–78
31. Vita, C., Dalzoppo, D. & Fontana, A. (1983) *Int. J. Peptide Protein Res.* **21,** 49–56
32. Vita, C., Dalzoppo, D. & Fontana, A. (1984) *Biochemistry* **23,** 5512–5519
33. Dalzoppo, D., Vita, C. & Fontana, A. (1985) *Biopolymers* **24,** 767–782
34. Dalzoppo, D., Vita, C. & Fontana, A. (1985) *J. Mol. Biol.* **182,** 331–340
35. Laemmli, U.K. (1970) *Nature* **227,** 680–685
36. Vita, C., Dalzoppo, D., Patti, S. & Fontana, A. (1984) *Int. J. Peptide Protein Res.* **24,** 104–111
37. Toniolo, C., Fontana, A. & Scoffone, E. (1975) *European J. Biochem.* **50,** 367–374
38. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954
39. Vita, C., Fontana, A. & Jaenicke, R. (1989) *European J. Biochem.* **183,** 513–518
40. Mitchinson, C. & Baldwin, R.L. (1986) *Proteins* **1,** 23–33
41. Schoemaker, K.R., Kim, P.S., York, E.J., Stewart, J.M. & Baldwin, R.L. (1987) *Nature* **326,** 563–567
42. Marqusee, S. & Baldwin, R.L. (1987) *Proc. Natl. Acad. Sci US* **84,** 8898–8902
43. Strehlow, K.G. & Baldwin, R.L. (1989) *Biochemistry* **28,** 2130–2133
44. Hecht, M.H., Sturtevant, J.M. & Sauer, R.T. (1986) *Proteins* **1,** 43–46
45. Imanaka, T., Shibazaki, M. & Takagi, M. (1986) *Nature* **334,** 695–697
46. Nicholson, H., Becktel, W.J. & Matthews, B.W. (1988) *Nature* **336,** 651–656
47. Sali, D., Byroft, M. & Fersht, A.R. (1988) *Nature* **335,** 740–743
48. Juillerat, M. & Homandberg, G.A. (1981) *Int. J. Peptide Protein Res.* **18,** 335–342
49. Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. & Zambonin, M. (1986) *Biochemistry* **25,** 1847–1851
50. Fontana, A. (1989) in *Highlights in Modern Biochemistry* (Kotyk, A., Skoda, J., Pachek, C. & Kostka, V., eds.), Vol. 2, pp. 1711–1726, VSP, Zeist
51. Wetlaufer, D.B. (1981 *Adv. Protein Chem.* **34,** 61–92
52. Rose, G.D. (1979) *J. Mol. Biol.* **134,** 447–470
53. Zehfus, M.H. (1987) *Proteins* **1,** 90–110
54. Janin, J. & Wodak, S.J. (1983) *Progr. Biophys. Mol. Biol.* **42,** 21–78
55. Jaenicke, R. (1987) *Progr. Biophys. Mol. Biol.* **49,** 117–237

Address:

Angelo Fontana
Department of Organic Chemistry
University of Padua
Via Marzolo 1
35131 Padua
Italy