Limited Proteolysis of Thermolysin by Subtilisin: Isolation and Characterization of a Partially Active Enzyme Derivative

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ABSTRACT: Incubation of the neutral metalloendopeptidase thermolysin at pH 9–10 in the presence of 10 mM $CaCl_2$ for 2 days at room temperature with subtilisin at a 50:1 molar ratio leads to a derivative possessing lower (~3%) but intrinsic catalytic activity. This derivative, called thermolysin S, was isolated by gel filtration in ~80% yield and then separated from some residual intact thermolysin by an affinity chromatographic step on Sepharose–Gly-D-Phe. It was found that thermolysin S results from a tight association of two polypeptide fragments of apparent M_r of 24000 and 10000. Dissociation of the complex was achieved under strong denaturing conditions, such as gel filtration on a column equilibrated and eluted with 5 M guanidine hydrochloride. The positions of the clip sites were defined by amino acid analysis, end-group determination, and amino acid sequencing of the isolated fragments and shown to lie between Thr-4 and Ser-5, between Thr-224 and Gln-225, and also between Gln-225 and Asp-226. Thermolysin S, which is therefore a stable complex of fragments 5–224(225) and 225(226)–316, shows a shift in optimum pH of about 1 unit toward the acid range with respect to intact thermolysin and a K_m essentially unchanged, with furylacryloyl-Gly-Leu-NH_2 as substrate. Inhibitors of thermolysin such as ethoxyformic anhydride and Zn^{2+} ions inactivate also the nicked enzyme. The overall conformational properties of thermolysin S appear very similar to those of the parent native enzyme, as judged by circular dichroism measurements and by the fact that rabbit antiserum against native thermolysin recognizes and precipitates thermolysin S, as detected by immunodiffusion. The lower activity of thermolysin S with respect to thermolysin can be related to the fact that cleavage occurs near the Asp-226 residue interacting with His-231, involved in the catalytic function of the enzyme. Inspection of the three-dimensional structure of thermolysin obtained crystallographically reveals that subtilisin cleaves at exposed loops of the protein molecule; in addition, the sites of cleavage are characterized by highest mobility in native thermolysin [Holmes, M. A., & Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623–639].

Limited proteolysis of protein molecules has been often employed to produce "nicked protein" species of functional and structural interest (Neurath, 1980). The most classical example is the proteolysis of ribonuclease A by subtilisin to produce the stable, active complex ribonuclease S, given by a tight association of the S peptide and S protein (Richards & Withayathil, 1959). A number of other nicked enzymes have been described in the literature, and these noncovalent complexes (complementing systems) have been quite useful in elucidating structure–function relationships of the parent, intact enzymes (Anfinsen & Scheraga, 1975). Often, it has been found possible to achieve reversible dissociation of nicked enzymes, so that chemical manipulation of the individual fragments allowed one to obtain modified complexes that have been studied with regard to enzyme activity and conformational stability (Baldwin & Creighton, 1980; Chaiken, 1981). Renewed interest in such complementing systems derives also from the fact that it is possible to achieve "proteolytic resynthesis" of the cleaved peptide bond by introducing an organic cosolvent into the reaction mixture (Homandberg et al., 1978, 1982).

This study was undertaken to learn if limited proteolysis might give some useful informations on the structure and function of thermolysin and, eventually, to characterize a nicked species to be subsequently used for the purposes outlined above. Thermolysin is a neutral metalloendopeptidase isolated from *Bacillus thermoproteolyticus* characterized by a remarkable stability to heat and protein denaturants (Endo, 1962; Matsubara & Feder, 1971; Ohta, 1967; Dahlquist et al., 1976; Fontana et al., 1977). The enzyme contains a catalytically essential zinc atom and four calcium ions, which play a prominent role in protein structure and stability (Roche & Voordouw, 1978). Extensive studies have been carried out on this enzyme, and the amino acid sequence (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972, 1974; Colman et al., 1972; Holmes & Matthews, 1982) have been determined. In the present work, thermolysin was submitted to limited proteolysis by subtilisin and the product of proteolysis examined. A nicked, partially active thermolysin derivative, designated thermolysin S,[1] was isolated, and its functional and structural properties were investigated.[2] This represents a continuation of our studies on the folding properties of thermolysin fragments (Vita et al., 1979, 1982, 1983; Vita & Fontana, 1982; Fontana et al., 1983).

Experimental Procedures

Materials. Thermolysin from *Bacillus thermoproteolyticus* (Rokko) was obtained from Calbiochem (San Diego, CA) as a lyophilized product containing 30% calcium and sodium

[1] Abbreviations: thermolysin S, nicked, partially active derivative of thermolysin constituted by the two-fragment complex [5–224(225)]–[225(226)–316]; ThS-1 and ThS-2, amino-terminal 5–224(225) and carboxyl-terminal 225(226)–316 fragments of thermolysin, respectively; FAGLA, N^{α}-(furylacryloyl)glycyl-L-leucine amide; Gdn-HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; MES, 2-(*N*-morpholino)ethanesulfonate; TCA, trichloroacetic acid; CNBr, cyanogen bromide; CD, circular dichroism.

[2] A preliminary report of this study was presented at the 15th FEBS Meeting, Brussels, Belgium, July 24–29, 1983, Communication S-05 TH-195.

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acetate. The enzyme was purified by affinity chromatography by using an affinity matrix of glycyl-D-phenylalanine covalently attached to aminoalkyl-Sepharose (Pangburn et al., 1973; Walsh et al., 1974). The resin employed contained 5 μmol of phenylalanine/mL of settled gel. The active enzyme was stored at −20 °C in 20 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$. A sample of trichloroacetic acid (TCA) inactivated protein was prepared by adding a ~1% solution (10 mL) of native thermolysin to a 2% TCA solution in water (50 mL) under vigorous stirring. The precipitate was collected by centrifugation, washed with water, and lyophilized.

Subtilisin Carlsberg, carboxypeptidase Y, and the proteins used as molecular weight markers for polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO). Tris, MES, SDS, cyanogen bromide, and *o*-phenanthroline were obtained from Fluka (Basel, Switzerland). FAGLA was purchased from Vega-Fox (San Diego, CA). Ultrapure guanidine hydrochloride (Gdn·HCl) (sequanal grade) was obtained from Pierce Chemical Co. (Rockford, IL), Sephacryl S-200 and Sephadex G-25 were from Pharmacia (Uppsala, Sweden), and Ultrogel AcA-54 was from LKB (Bromma, Sweden). All other chemical compounds were of analytical grade and were purchased from C. Erba (Milan, Italy) or Merck AG (Darmstadt, Germany).

Conditions of Proteolysis. To a solution (1 mg/mL) of thermolysin purified by affinity chromatography in 10 mL of 50 mM Tris-HCl buffer–10 mM $CaCl_2$, containing 5 mM *o*-phenanthroline, was added subtilisin Carlsberg (2% w/w) under stirring. Incubation experiments were carried out in the pH range 7–10, at 37 °C. A control of thermolysin was kept at pH 9.0, at 37 °C, without subtilisin. At intervals, aliquots were removed from the reaction mixture and assayed for proteolytic activity with the standard assay mixture (see below) containing 10^{-5} M $ZnCl_2$. Samples were also taken from the reaction mixture for SDS electrophoresis.

A relatively large-scale preparation of nicked thermolysin was prepared by dissolving commercial thermolysin (1 g, containing about 10% of autolytic products and 30% salt) in 600 mL of 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, and 5 mM *o*-phenanthroline, pH 9.0, in the presence of 12 mg of subtilisin Carlsberg. The reaction mixture was gently stirred at 37 °C for 2 days.

Isolation of Thermolysin S. An aliquot of the proteolytic mixture (~5% residual thermolytic activity) (15 mg of protein in 15 mL of Tris buffer, pH 9.0) was applied to an Ultrogel AcA-54 column (3.7 × 80 cm) equilibrated with 20 mM Tris-HCl buffer, pH 9.0, containing 10 mM $CaCl_2$. Chromatography was carried out at 5 °C. A protein peak coincident with activity was eluted first from the column, followed by a peak containing peptide material of low molecular weight and *o*-phenanthroline. The fractions containing the protein were pooled, dialyzed against a solution of 10 mM MES buffer, pH 6.0, containing 10 mM $CaCl_2$ and 10^{-5} M $ZnCl_2$, and concentrated with an Amicon apparatus using a YM-5 membrane, M_r cutoff 5000. This solution was applied to a Sepharose–Gly-D-Phe (Pangburn et al., 1973; Walsh et al., 1974) column, equilibrated with the same MES buffer, pH 6.0, but without zinc added. Elution was carried out in two steps with 20 mM Tris-HCl buffer, pH 7.0, and then with 0.1 M Tris-HCl buffer, pH 9.0, both containing 10 mM $CaCl_2$.

Isolation of Proteolytic Fragments. A 3-mL solution (~2 mg/mL) of thermolysin S (as obtained from the affinity column) was poured dropwise under stirring into 15 mL of a 10% aqueous formic acid solution. The mixture was then lyophilized and the residue dissolved in 1.5 mL of 5 M Gdn·HCl–50 mM sodium acetate, pH 5.0. This solution was applied to a Sephacryl S-200 column (1.8 × 80 cm), equilibrated and eluted with this Gdn·HCl solution. The effluent from the column was monitored at 280 nm. Two well-separated peaks of peptide material were eluted from the column. The fractions corresponding to these peaks were combined separately and extensively dialyzed against 2% aqueous formic acid. The peptide material from the first peak precipitated during dialysis and was separated by centrifugation. The two peptide samples were then lyophilized.

Amino Acid Analysis. Proteins and peptides were hydrolyzed in 6 N HCl at 110 °C for 22 h in tubes sealed under vacuum. Hydrolyzates were analyzed on a Jeol Model JLC-6AH amino acid analyzer using the single-column methodology, according to the manufacturer's instructions.

Sequence Determination. Automatic Edman degradation was performed with a Beckman Sequencer Model 890B (updated), using quadrol buffer system and the Beckman peptide program (060275) by Dr. K. J. Wilson (Institute of Biochemistry, University of Zurick, Zurick, Switzerland). Amino acid phenylthiohydantoin derivatives were identified by amino acid analysis on a Durrum D-500 analyzer after acid hydrolysis in 6 N HCl–0.1% stannous chloride, 150 °C, 4 h (Mendez & Lai, 1975). Alternatively, degradation cycles were carried out manually with the phenyl isothiocyanate reagent following essentially the procedure of Tarr (1982). Phenylthiohydantoins of amino acids were identified by reverse-phase high-pressure liquid chromatography with a Perkin-Elmer Model 3B instrument using a C-18 Biosil ODS-5S column (Bio-Rad, Richmond, CA). The mobile phase was 10 mM sodium acetate, pH 4.1, and the modifier was acetonitrile–methanol (4:1 v/v). The concentration of the modifier was increased linearly from 15 to 50% in 20 min at a flow rate of 1 mL/min. The effluent was monitored at 266 nm.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out in a vertical slab gel apparatus (Laemmli, 1970). An exponential gradient from 15 to 24% along the direction of migration was used. The gels (1-mm thickness) were strained with Coomassie Brilliant Blue R-250.

Enzyme Activity. The activity of thermolysin was measured by monitoring the decrease of FAGLA absorbance at 345 nm at 25 °C. The assay solution (3 mL) contained FAGLA (1 mM) in 50 mM HEPES buffer, pH 7.0, 10 mM $CaCl_2$, and the catalytic reaction was initiated by adding an enzyme solution (5–50 μL). The decrease in absorbance at 345 nm was followed as a function of time.

Metal Analyses. The zinc content of thermolysin and thermolysin S was determined by a Hitachi Perkin-Elmer Model 360 atomic absorption spectrometer. Thermolysin and thermolysin S (~1 mg/mL) were dissolved in 20 mM Tris-HCl–10 mM $CaCl_2$ in the presence of 10^{-5} M $ZnCl_2$ and then gel-filtered on a Bio-Gel P-2 column equilibrated and eluted with the same buffer without zinc added.

Protein Concentration. The concentration of both thermolysin and thermolysin S was determined from absorbance measurements at 280 nm by using $A^{1\%}_{280nm}$ = 15.2 (Voordouw & Roche, 1974).

Spectroscopic Measurements. CD measurements were performed as outlined previously (Vita et al., 1979). Absorbance values at single wavelengths and continuous absorption spectra were obtained with a Hitachi Perkin-Elmer Model 554 spectrophotometer. Fluorescence measurements were carried out with a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer (Fontana et al., 1977).

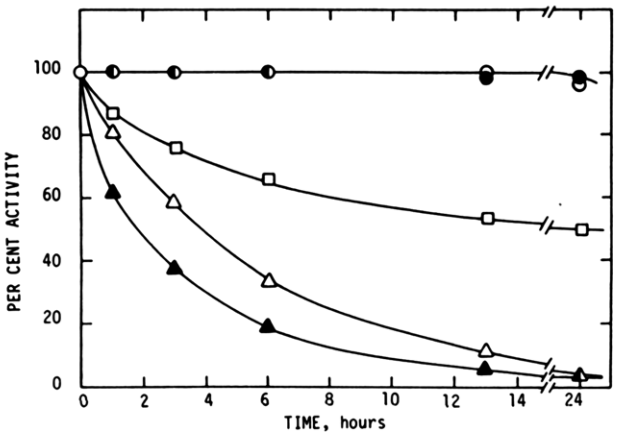

FIGURE 1: Kinetics of the limited digestion of thermolysin by subtilisin. Thermolysin was incubated (1 mg/mL) at 37 °C in 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, and 5 mM *o*-phenanthroline, in the presence of subtilisin (2:100 w/w). The pH of the solution was 7.0 (○), 8.0 (□), 9.0 (△), and 10.0 (▲). As a control, thermolysin was incubated under the same conditions at pH 9.0, 37 °C, without subtilisin (●). At the indicated times, a sample of the reaction mixture was assayed for proteolytic activity with FAGLA as substrate. The assay mixture contained 10^{-5} M $ZnCl_2$ (see Experimental Procedures).

Immunochemical Techniques. Double-immunodiffusion experiments were carried out with rabbit anti-thermolysin serum (Vita et al., 1979) in 1% agar in 20 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 10 mM $CaCl_2$, according to the method of Ouchterlony (1948). After 1 day at room temperature in a humidified chamber, the agar gel was washed with 5% NaCl solution and then distilled water, dried, and then stained with 1% Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (45:10:45 v/v/v).

RESULTS

Kinetics of Proteolysis. The proteolysis of thermolysin by subtilisin has been followed by monitoring the thermolysin activity with FAGLA as substrate. The proteolytic digestion was carried out in Tris buffer, containing 10 mM $CaCl_2$ and 5 mM *o*-phenanthroline. It was found useful to include this chelating agent in the reaction mixture in order to inactivate thermolysin by removal of its functional zinc ion (Latt et al., 1969; Holmquist & Vallee, 1974) and thus to prevent autolysis. The results shown in Figure 1 indicate that incubation of thermolysin with subtilisin (2% w/w) at 37 °C leads to a progressive loss of thermolytic activity and that the rate of inactivation is faster at mildly alkaline pH. At pH 7, thermolysin appears completely stable to the action of subtilisin. For preparative purposes, the incubation was allowed to proceed until ~5% of the original thermolytic activity remained, after which the proteolytic mixture was worked up as described below.

When aliquots of the incubation mixture containing thermolysin and subtilisin were subjected to polyacrylamide gel electrophoresis in the presence of SDS (Laemmli, 1970), it was observed that as the time of incubation increased there was a gradual disappearance of the band corresponding to thermolysin (M_r 34 000) and the appearance of two more rapidly migrating bands (Figure 2). On the other hand, when thermolysin was incubated under identical conditions without subtilisin, a single band of intact protein was observed in the gel. Using proteins of known molecular weight as markers, it was found that the two fragments have M_r of approximately 24 000 and 10 000. The larger fragment will be herewith designated ThS-1 and the smaller one ThS-2. The observed molecular weight of the two fragments would indicate that thermolysin is cleaved in just two pieces. However, it is known

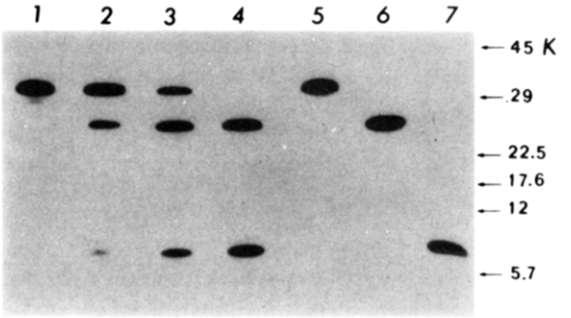

FIGURE 2: Limited proteolysis of thermolysin by subtilisin monitored by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) of digestion products. During proteolysis of thermolysin under the conditions reported in the legend of Figure 1 (pH 9.0, 37 °C), samples were taken from the reaction mixture immediately after the addition of subtilisin (1) and after 1 (2), 3 (3), and 24 h (4) of incubation. Sample 5 corresponds to thermolysin incubated for 24 h under the same conditions, but without subtilisin. Samples 6 and 7 correspond to the isolated NH_2- and COOH-terminal fragments, respectively. The marker proteins in order of decreasing molecular weight are ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, myoglobin, cytochrome *c*, and insulin.

that small molecular weight peptides, if present in the proteolytic mixture, remain undetected in SDS–polyacrylamide electrophoresis gels (see below).

Isolation of Thermolysin S. Chromatography on an Ultrogel AcA-54 column of an aliquot of the proteolytic mixture (~5% residual thermolytic activity) yielded a major protein peak located at the same position as that of intact thermolysin, followed by a minor peak of low molecular weight material. Of note, the protein possessed thermolytic activity when Zn^{2+} ions were added in the standard assay mixture. When an aliquot of this protein material was subjected to SDS gel electrophoresis, the two bands corresponding to fragments ThS-1 and ThS-2 were detected, with just a faint band of intact thermolysin. These results clearly indicate that the fragments in the nicked protein remain associated under the conditions employed for gel filtration.

In order to separate residual intact thermolysin from the nicked protein (herewith called thermolysin S), the protein solution obtained from the gel filtration step was dialyzed against 10 mM MES buffer, pH 5.8, containing 10 mM $CaCl_2$ and 10^{-5} M $ZnCl_2$, and applied to a Sepharose–Gly-D-Phe column. This affinity matrix was found to bind strongly native thermolysin at neutral pH, the absorbed enzyme being then eluted from the column at pH 9 (Pangburn et al., 1973; Walsh et al., 1974). Figure 3 shows a typical elution profile from the affinity column of a sample of the nicked protein obtained after the gel filtration step (see above). It is seen that essentially all the protein material applied to the column remains bound to the affinity matrix at pH 5.8 and that two peaks of active material are eluted at pH 7 and 9. In separate experiments, it was found that subtilisin is eluted at pH 5.8 from the affinity column. SDS gel electrophoresis clearly indicated that the protein material eluted at pH 7 and 9 corresponded to the nicked protein and to intact thermolysin, respectively.

Initial experiments of proteolysis were carried out with samples of thermolysin highly purified by affinity chromatography on Sepharose–Gly-D-Phe (see Experimental Procedures). Yields of thermolysin S, after the two chromatographic steps, were over 80%. Subsequently, it was found convenient to prepare thermolysin S by employing directly commercial samples of thermolysin, containing some 10% autolytic products. A relatively large-scale preparation of thermolysin S was obtained by subjecting to proteolysis with subtilisin 1 g of protein powder (containing 30% salt) for 2 days at 37 °C in

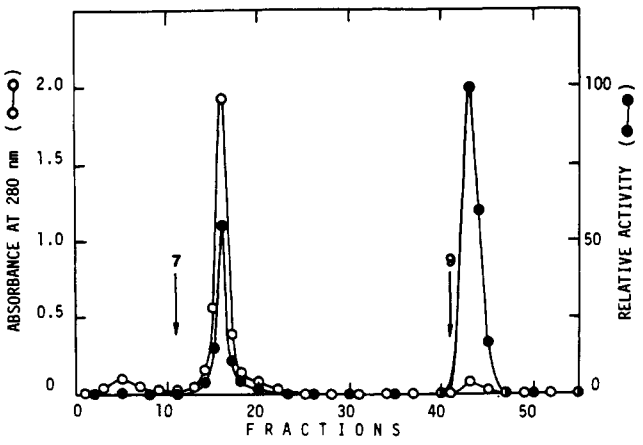

FIGURE 3: Affinity chromatography on a Sephadex–Gly-D-Phe column of a mixture of nicked and intact thermolysin, as obtained after gel filtration (see Experimental Procedures). The affinity column (1 × 6 cm), equilibrated at 5 °C with 10 mM MES buffer, pH 6.0, containing 10 mM $CaCl_2$, was eluted first with the equilibrating buffer, then with 20 mM Tris-HCl, pH 7.0, and finally with 0.1 M Tris-HCl, pH 9.0, both buffers containing 10 mM $CaCl_2$. Fractions of 3 mL were collected at a flow rate of 10 mL/h and analyzed for protein content at 280 nm (○) and proteolytic activity (●).

50 mM Tris-HCl buffer, pH 9.0, containing 10 mM $CaCl_2$ and 5 mM *o*-phenanthroline. The yield of purified thermolysin S under these conditions was ~75%.

Since thermolysin is a metalloenzyme with a zinc ion located at the active site (Latt et al., 1969; Matthews et al., 1972), the content of this metal in the two protein samples as obtained after the affinity chromatography step (see Figure 3) was evaluated by atomic absorption measurements. Both thermolysin and thermolysin S contained 1 equiv (0.9–1.05) of zinc/mol of protein.

Isolation of Protein Fragments and Elucidation of the Cleavage Sites. To achieve separation of the fragments ThS-1 and ThS-2 constituting the complex of thermolysin S, the purified nicked protein was denatured as described under Experimental Procedures and then passed through a Sephacryl S-200 column equilibrated and eluted with 5 M Gdn·HCl in 50 mM acetate buffer, pH 5.0. The elution pattern from this column, shown in Figure 4, gave two rather well separated protein peaks. The relative concentration of the fragments cannot be judged by the absorption at 280 nm, since they differ in tryptophan content (see below). On the basis of the elution position from the Sephacryl S-200 column, the approximate M_r of the protein material of the first and second peak was established as 24 000 and 10 000, respectively, with proteins of known molecular weight as markers (Figure 4, insert). Thus, the molecular weights of fragments ThS-1 and ThS-2 established either by gel filtration or by SDS gel electrophoresis (above) were in good agreement.

To characterize the isolated fragments ThS-1 and ThS-2, their absorption and fluorescence spectra were recorded. Absorption spectra of the fragments dissolved in 5% aqueous acetic acid showed maxima of absorption at 275–280 nm, as would be expected for polypeptides containing both tyrosine and tryptophan. A shoulder at 290 nm, typical of tryptophan residues, was evident in the spectrum of fragment ThS-1. By use of an excitation wavelength of 280 nm, ThS-1 showed a fluorescence emission spectrum typical of tryptophan-containing polypeptides, with a maximum near 350 nm (Brand & Witholt, 1967). Fragment ThS-2 showed only tyrosine fluorescence emission, with a maximum near 303 nm and no peaks or shoulders near 350 nm. Considering that the three tryptophan residues in intact thermolysin are located in pos-

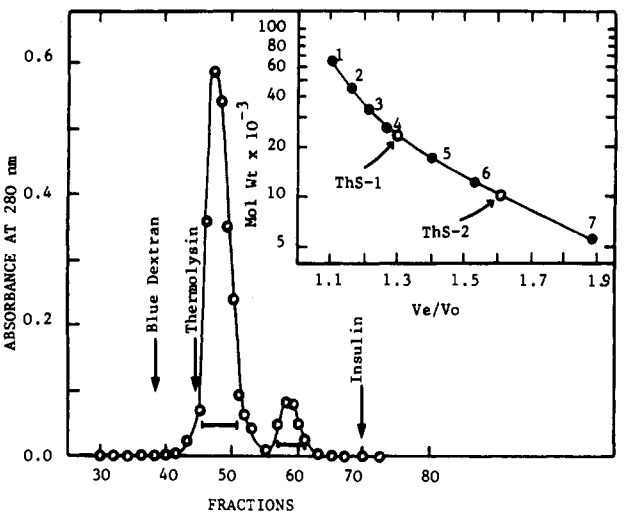

FIGURE 4: Gel filtration of thermolysin S on Sephacryl S-200 under denaturing conditions. A sample of thermolysin S (~7 mg) was dissolved in 2 mL of 5 M Gdn·HCl and 50 mM acetate buffer and directly applied to a Sephacryl S-200 column (1.8 × 80 cm) equilibrated with the same Gdn·HCl solution. Fractions of 2 mL were collected at a flow rate of 10 mL/h and analyzed at 280 nm for protein content. (Insert) Molecular weight estimation of the fragments (ThS-1 and ThS-2, high and low molecular weight fragments, respectively) produced by limited proteolysis of thermolysin by elution position from the Sephacryl S-200 column. The marker proteins were (1) bovine serum albumin, (2) ovalbumin, (3) intact thermolysin, (4) carbonic anhydrase, (5) myoglobin, (6) horse heart cytochrome *c*, and (7) insulin. Horizontal bars indicate fractions pooled.

itions 55, 115, and 186 of the polypeptide chain, it can be concluded that ThS-1 and ThS-2 derive from the NH_2- and COOH-terminal portion of the thermolysin chain, respectively.

Determination of the sites of the subtilisin cleavage of thermolysin was obtained by NH_2-terminal sequence analysis of fragments ThS-1 and ThS-2. The Edman degradation cycles with phenyl isothiocyanate were carried out both manually and with the aid of the sequenator (see Experimental Procedures). Five degradation cycles established that fragment ThS-1 has the unique NH_2-terminal sequence Ser-Thr-Val-Gly-Val. Since this sequence corresponds to residues 5–9 of intact thermolysin (Titani et al., 1972), the results of sequence analysis indicate that ThS-1 results from cleavage of thermolysin at the single site Thr^4-Ser^5 in the NH_2-terminal region of the polypeptide chain. As a control, a sample of trichloroacetic acid (TCA) inactivated thermolysin, prepared as described under Experimental Procedures, was subjected to five degradation cycles in the sequenator. The results clearly indicated sequence Ile-Thr-Gly-Thr-Ser.

With fragment ThS-2, each cycle of the sequencing procedure yielded two amino acid residues in roughly equal amounts (after conversion of phenylthiohydantoins to free amino acids by acid hydrolysis): Glx and Asp (first cycle), Asx (second), Asx and Gly (third), Gly (fourth), Gly and Val (fifth). Only traces of other amino acids were obtained. Considering that segment 224–230 in the intact protein is -Thr-Gln-Asp-Asn-Gly-Gly-Val-, these results of sequence analysis are consistent with fragment ThS-2 being a mixture of two peptides starting with Gln^{225} and Asp^{226}.

In order to check if cleavage of thermolysin with subtilisin occurred at the COOH terminus, the carboxyl-terminal sequence of fragment ThS-2 was established by carboxypeptidase Y digestion. The results obtained clearly indicated the integrity of the COOH terminus of ThS-2, since the kinetics of the amino acid released upon incubation of ThS-2 with the exopeptidase was consistent with the known amino acid sequence

Table I: Amino Acid Composition of Thermolysin, Thermolysin S, and Thermolysin Fragments[a]

amino acid	thermolysin	thermolysin S	ThS-1 [5–224(225)]	ThS-2 [225(226)–316]	sum of columns 4 and 5
aspartic acid	44.9 (44)	44.5 (44)	35.7 (36)	7.9 (8)	43.6 (44)
threonine	25.3 (25)	22.9 (23)	16.3 (17)	5.8 (6)	22.1 (23)
serine	25.2 (26)	24.5 (26)	16.0 (17)	9.0 (9)	25.0 (26)
glutamic acid	21.7 (21)	22.4 (21)	14.2 (13–14)	7.5 (7–8)	21.7 (21)
proline	8.8 (8)	7.8 (8)	7.2 (7)	1.2 (1)	8.4 (8)
glycine	36.7 (36)	35.0 (35)	24.2 (24)	10.8 (11)	35.0 (35)
alanine	28.0 (28)	29.0 (28)	18.4 (18)	9.9 (10)	28.3 (28)
valine	19.6 (22)	21.3 (22)	12.6 (13)	8.5 (9)	21.1 (22)
methionine	1.8 (2)	2.2 (2)	2.2 (2)	0.0 (0)	2.2 (2)
isoleucine	16.5 (18)	16.4 (17)	12.2 (11)	5.1 (6)	17.3 (17)
leucine	14.9 (16)	16.4 (16)	10.7 (10)	6.1 (6)	16.8 (16)
tyrosine	26.1 (28)	27.2 (28)	21.1 (23)	4.9 (5)	26.0 (28)
phenylalanine	9.2 (10)	9.8 (10)	7.2 (7)	3.0 (3)	10.2 (10)
lysine	10.8 (11)	11.4 (11)	6.3 (6)	4.4 (5)	10.7 (11)
histidine	7.2 (8)	7.5 (8)	5.8 (6)	2.0 (2)	7.8 (8)
arginine	9.3 (10)	10.4 (10)	7.0 (7)	3.1 (3)	10.1 (10)
tryptophan[b]	+ (3)	+ (3)	+ (3)	(0)	– –

[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 (Titani et al., 1972). The figures given are average values obtained from three separate amino acid analysis after 22-h acid hydrolysis and without correction for destruction or low recovery during hydrolysis. The NH_2- and COOH-terminal protein fragments ThS-1 and ThS-2 were prepared as described in the text. [b] The tryptophan content was determined qualitatively by fluorescence emission at 350 nm upon excitation at 295 nm of protein or peptide solutions in 6 M Gdn·HCl (Brand & Witholt, 1967).

of thermolysin at its COOH terminus (Titani et al., 1972) (see Table I of supplementary material; see paragraph at end of paper regarding supplementary material).

The results of the sequence analysis of both fragments ThS-1 and ThS-2 were also confirmed by the isolation of the cyanogen bromide (CNBr) fragments of thermolysin S. To this aim, homogeneous thermolysin S (as obtained after affinity chromatography, Figure 3) was cleaved with CNBr under the same experimental conditions previously employed to cleave intact thermolysin at the level of the two methionine residues in positions 120 and 205 of the polypeptide chain of 316 amino acid residues (Titani et al., 1972; Vita et al., 1979). From the CNBr reaction mixture of thermolysin S, fragments 5–120, 121–205, 206–224(225), and 225(226)–316 were isolated and characterized (for experimental details, see supplementary material). In particular, the amino acid composition of fragment 206–224(225) gave 0.6 mol/mol of fragment of the COOH-terminal glutamic acid (Table III of supplementary material), in agreement with the expected heterogeneity of fragment ThS-1 at its COOH terminus.

The amino acid composition of thermolysin S and fragments ThS-1 and ThS-2 are shown in Table I. The composition of thermolysin is also shown for comparison. The sum of the compositions of the two separated fragments ThS-1 and ThS-2 corresponds well to that of thermolysin S, again indicating that the two fragments are the constituting species of the nicked protein.

Taken together, these results indicate that subtilisin cleaves thermolysin, under the conditions above described, at the single peptide bond Thr^4–Ser^5 in the NH_2-terminal part of the molecule and at the two peptide bonds Thr^{224}–Gln^{225} and Gln^{225}–Asp^{226} in the middle part. Thus, the peptide fragments ThS-1 and ThS-2 are the constituting species of the thermolysin S complex. The possibility that purified thermolysin is a three-fragment complex (ThS-1, ThS-2, and the tetrapeptide 1–4) seems to be excluded by the data of the amino acid composition of thermolysin S (Table I) and by the fact that an Edman degradation cycle of thermolysin S did not revealed the presence of the phenylthiohydantoin of isoleucine (the NH_2-terminal residue of thermolysin).

Enzymological Properties of Thermolysin S. When FAGLA was used as substrate, thermolysin S showed an optimum of activity at pH 5.5–6.0, being about 1 unit less than

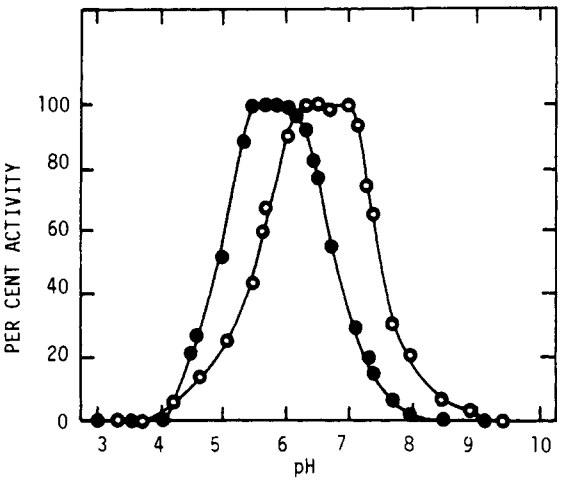

FIGURE 5: Effect of pH on the activity of thermolysin (○) and thermolysin S (●)., Assays were performed at 25 °C with FAGLA as substrate at 1 mM concentration in sodium acetate (pH range 3–5.3), MES (pH range 5.5–7.0), and Tris-HCl buffer (pH range 7–9.5). All buffers were 50 mM and contained 10 mM $CaCl_2$ and 10^{-5} M $ZnCl_2$. The enzyme concentration in the assay mixtures was 4.1×10^{-8} M for thermolysin and 4.3×10^{-6} M for thermolysin S.

that of thermolysin (Figure 5). The values of K_m [$(2.4–2.7) \times 10^{-3}$ M], estimated from Lineweaver–Burk plots, were similar for both at pH 6 and 7, whereas the specific activity of thermolysin S is significantly less than that of thermolysin, since at pH 6.0 thermolysin S maintains ~3% activity.

Thermolysin S shares in common with the parent enzyme the property of being inactivated by low concentrations of Zn^{2+} ions (Holmquist & Vallee, 1974) and ethoxyformic anhydride (Burstein et al., 1974; Pangburn et al., 1976). The inactivation of thermolysin by ethoxyformic anhydride was shown previously to be the result of modification of a single histidine residue, most likely His-231; reactivation can be accomplished by incubation of the ethoxyformylated enzyme with hydroxylamine (Burstein et al., 1974; Pangburn & Walsh, 1975). As shown in Figure 6, when the enzymes are assayed for enzymatic activity with FAGLA as substrate at pH 6.0 in the presence of Zn^{2+} ions, approximately half inactivation occurs at 10^{-2} and 10^{-3} M zinc concentration for thermolysin and thermolysin S, respectively. The rates of both inactivation with ethoxyformic anhydride and reactivation with hydroxylamine

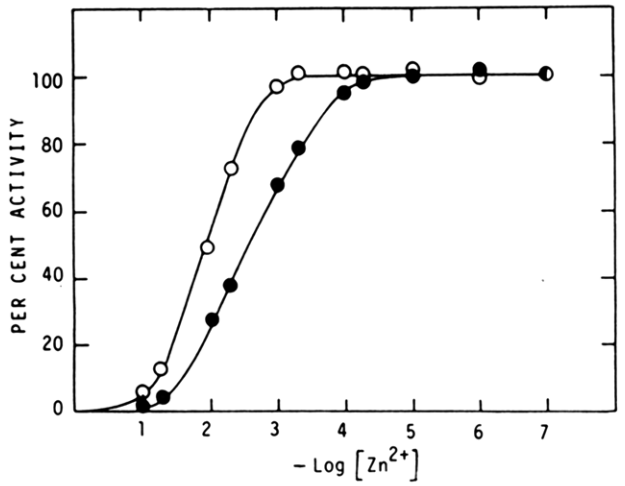

FIGURE 6: Inhibition of thermolysin (○) and thermolysin S (●) by zinc ions. Enzymatic assays were conduced at 22 °C in 0.1 M MES buffer, pH 6.0, containing 10 mM $CaCl_2$ and the indicated concentrations of zinc. FAGLA was used as substrate, and activity was expressed in terms of initial velocity of substrate hydrolysis. The results are expressed in terms of percent activity with respect to that measured without zinc added to the assay solution. The enzyme concentration in the assay mixture was 4.5×10^{-8} M for thermolysin and 6.1×10^{-6} M for thermolysin S.

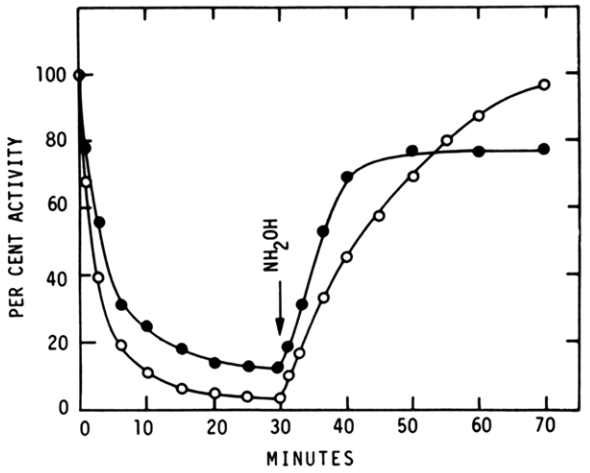

FIGURE 7: Inactivation of thermolysin (○) and thermolysin S (●) by ethoxyformic anhydride. Thermolysin was reacted at 22–23 °C with 4.3 mM ethoxyformic anhydride in 20 mM Tris-HCl–10 mM $CaCl_2$ buffer, pH 7.0, at a protein concentration of 0.5 mg/mL. Reactivation was accomplished by addition of hydroxylamine to a final concentration of 20 mM (pH 7.2). Thermolysin S was similarly reacted in 0.1 M MES–10 mM $CaCl_2$ buffer, pH 6.0, at a protein concentration of 0.9 mg/mL with 0.43 mM ethoxyformic anhydride. Reactivation was accomplished with 0.13 M hydroxylamine (pH 6.2).

were found quite different for thermolysin and thermolysin S, and these were found comparatively similar under the specific experimental conditions of pH and reagent concentration indicated in the legend to Figure 7. In particular, at pH 7.0, thermolysin S reacts with ethoxyformic anhydride much faster than thermolysin, indicating the presence of a more reactive His-231; at pH 6.0, the rate of inactivation of thermolysin S is comparable to that observed for thermolysin at pH 7.0. These differences in behaviour toward both Zn^{2+} ions and ethoxyformic anhydride can be taken as supportive evidence of different p*K* values of His-231 in both enzymes, being more reactive, and thus more nucleophilic, the imidazole group in thermolysin S.

Circular Dichroism Studies. The far- and near-ultraviolet CD spectra of thermolysin and thermolysin S are shown in Figure 2 of the supplementary material. Below 250 nm, both

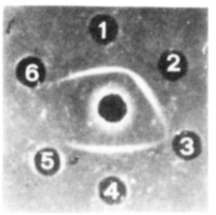

FIGURE 8: Double-immunodiffusion assays showing precipitin reactions of thermolysin and thermolysin S against rabbit anti-thermolysin serum 1% agar in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$. The center well contained 7 μL of serum, and the outer wells contained 7 μL of the following solutions in Tris buffer: (1) native thermolysin (0.7 mg/mL); (2 and 4) thermolysin S (0.7 mg/mL); (3) 2% bovine serum albumin; (5) 10 mM Tris buffer, pH 8.0, 10 mM $CaCl_2$; (6) heat-denatured thermolysin (0.2 mg/mL).

molecules exhibit the same minimum and intensities of ellipticity near 210 nm and a shoulder at 220 nm. In the region of the side-chain absorption, the spectra were the same for both proteins, with a negative trough centered at about 276 nm.

Immunochemical Studies. Double-immunodiffusion experiments (Ouchterlony, 1948) (Figure 8) show that both native thermolysin and thermolysin S recognize and precipitate rabbit anti-thermolysin antibodies (Vita et al., 1979). The smooth fusion between the precipitin lines induced by thermolysin and thermolysin S indicates that likely the nicked protein contains all the precipitin sites of the intact native protein.

Stability Studies. Active thermolysin S, with the full complement of Zn^{2+} ions, is relatively stable upon standing at room temperature for 90 min at pH 6–9 in the presence of 10 mM $CaCl_2$, whereas outside this pH range is irreversibly inactivated. This inactivation was shown by SDS–polyacrylamide gel electrophoresis to be the result of autolytic degradation. A comparison of the pH-stability curves of thermolysin S (Figure 3 of supplementary material) with those previously reported for thermolysin (Ohta, 1967) indicates that the nicked enzyme is less stable under acid or alkaline conditions than the parent intact enzyme. In order to prevent autolysis and thus inactivation, thermolysin S was stored in solution as the apoenzyme (zinc free).

The thermal stability of thermolysin S is quite high but less than that of thermolysin (Figure 4 of supplementary material). Upon heating for 10 min in 10 mM Tris-HCl buffer, pH 7.5, in the presence of 10 mM $CaCl_2$, a temperature of 68 and 76 °C is required for 50% inactivation of nicked and intact enzyme, respectively.

Discussion

The results presented here show that limited proteolysis of thermolysin can be effected by subtilisin, leading to a well-defined nicked protein species, thermolysin S, possessing lower but intrinsic enzymatic activity. Evidences have been obtained that thermolysin S is given by a strong association of the two polypeptide chains of ThS-1 and ThS-2, corresponding to segments 5–224(225) and 225(226)–316 of the thermolysin polypeptide chain, respectively. The fact that subtilisin cleaves peptide bonds Thr^{224}–Gln^{225} and Gln^{225}–Asp^{226} in the 220–230 region of thermolysin can be considered a result of the broad specificity of subtilisin (Harris & Roos, 1959) on one side and a highly accessible loop on the other. This multiple site of proteolysis by subtilisin is not new and is similar to that already observed with other proteins (Raibaud & Goldberg, 1973; Titani et al., 1977; Koide et al., 1978; Bloxham et al., 1980). In the case of ribonuclease A, the subtilisin-susceptible peptide bond is Ala^{20}–Ser^{21} (Richards & Withayathil, 1959) and to a lesser extent Ser^{21}–Ser^{22} (Gross & Witkop, 1967; Doscher

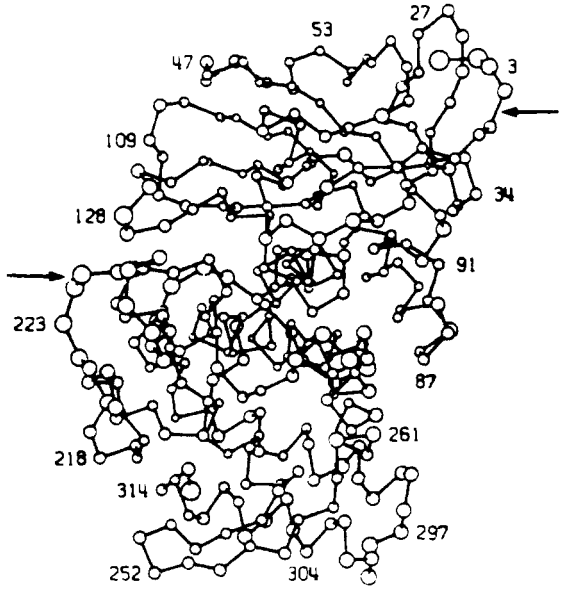

FIGURE 9: Backbone diagram of the thermolysin crystallographic structure. Larger circles correspond to residues with greater apparent motion. The arrows indicate the sites of cleavage by subtilisin [adapted with permission from Holmes & Matthews (1982)].

& Hirs, 1967) and these peptide bonds are situated in an exposed loop of the polypeptide chain (Kartha et al., 1967; Wyckoff et al., 1967). Figure 9 shows that indeed the sites of cleavage of thermolysin by subtilisin are exposed to the solvent.

Limited proteolysis of a globular protein can be attributed to both *exposure and flexibility* of the polypeptide chain at the site of attack, since this flexibility would allow the polypeptide substrate to be properly accomodated at the active site of the protease with concomitant peptide bond fission. This correlation between flexibility and proteolysis has been well documented in few cases, e.g., the autolysis loop of the trypsinogen–trypsin system and the hinge peptide between domains in immunoglobulins [see Huber & Bennett (1983) for references and a thorough discussion]. On this basis, it is of interest to relate the results of this study with the flexibility of the thermolysin polypeptide chain, recently determined by X-ray diffraction methods (Holmes & Matthews, 1982). Since it has been found that "highest mobility occurs for exposed surface loops in the vicinity of residues 128, 180, and 225, as well as the amino and carboxyl termini," it is quite interesting to find that indeed the two sites of cleavage of the chain of thermolysin by subtilisin are included among the sites characterized by high mobility (cf. Figure 9).

Optimal limited proteolysis of thermolysin occurs in alkali (Figure 1). At present, it is not clear if this can be related to the optimum pH for activity of subtilisin, since this protease shows an optimum pH range between 7 and 9 with synthetic substrates, 7–8 with denatured hemoglobin, and 10–11 with casein (Ottesen & Svendsen, 1970). Alternatively, it can be proposed that alkali induces in the substrate thermolysin a conformational transition to a more open form that is easily attacked by subtilisin. This is conceivable, considering the existence in the crystal structure of thermolysin of a salt bridge between the carboxylate group of Asp-226 and the imidazolium group of His-231 (Colman et al., 1972) and that it is possible that deprotonation of the imidazolium group in alkali results in elimination of this salt bridge, with concomitant local conformational change in the loop near 220–230. In actual fact, limited proteolysis of thermolysin by subtilisin was carried out in the presence of the zinc-specific chelating agent *o*-phenanthroline, i.e., with apothermolysin and not holothermolysin as substrate. However, spectroscopic measurements indicated that these two protein species possess similar three-dimensional structure, including thermostability (Holmquist & Vallee, 1974; Grandi et al., 1980). Matthews et al. (1974) demonstrated that crystals of zinc-free and native thermolysin are closely isomorphous and the difference in electron density between them indicates only slight adjustments in residues in the immediate vicinity of the zinc ion.

Several lines of evidence indicate that thermolysin S possesses intrinsic activity. First of all, the effective separation of nicked from intact thermolysin by affinity chromatography (Figure 3) indicates that it is unlikely that the thermolysin S sample herewith studied is contaminated by thermolysin. The observed differences between the two enzyme species in both optimum of pH for activity and thermal stability are taken as clear supportive evidence for an active thermolysin S. In addition, the inhibition studies using Zn^{2+} ions and ethoxyformic anhydride as inhibitors revealed that the nicked and intact enzyme behave differently (Figures 6 and 7). The lower specific activity of thermolysin S with respect to thermolysin can be related to analogous reduction of enzymatic activity observed with several other nicked enzymes already described in the literature, e.g., ribonuclease S (Takahashi et al., 1972), desoxyribonuclease (Hugli, 1972), carboxypeptidase (Cueni et al., 1980), and aspartate aminotransferase (Sandmeier & Christen, 1982). The low activity of thermolysin S can be easily interpreted considering that a clip site is generated near the Asp-226 specifically interacting with His-231 of the active site (Colman et al., 1972; Matthews et al., 1974; Pangburn & Walsh, 1975). That the charge relay at the active site of thermolysin S is changed and thus cannot be optimal is also reflected by the shift toward the acid pH range of the optimum of pH for activity (Figure 5).

Considering that thermolysin S maintains important functional properties of the intact native enzyme (activity, binding to an inhibitor, specific binding of the functional zinc ion), a similar overall three-dimensional structure between the nicked and intact protein was anticipated. Thus, CD spectra in the far-ultraviolet region of thermolysin S and thermolysin were similar, indicating an unchanged overall polypeptide backbone conformation (Chen et al., 1974). In the near-ultraviolet region, the CD spectra of both proteins were also similar, indicating that the aromatic residues are located in similar environments in both proteins (Strickland, 1974). These spectroscopic data of structure were also complemented by an immunochemical approach using rabbit anti-thermolysin antibodies (Vita et al., 1979). Immunodiffusion analysis indicated that the antigen–antibody interaction is apparently identical when either thermolysin or thermolysin S is used as antigen in these assays. Considering that antibodies elicited toward a globular protein are specific for antigenic determinants that are located in the more exposed regions (loops, corners) of the protein molecule (Atassi & Saplin, 1968; Sachs et al., 1972; Crumpton, 1974; Lazdunski, 1976; Reichlin, 1975; Habeeb, 1977), it can be concluded that important structural details of the three-dimensional structure are shared between thermolysin S and the parent native protein.

A particular interest of nicked proteins resides in the possibility they offer for investigating the process of protein folding (Zetina & Goldberg, 1982; Dautry-Varsat & Garel, 1981). In the present case, this will be possible if intact native thermolysin can be reversibly denatured on one side, if isolated fragments ThS-1 and ThS-2 can be renatured separately, and finally if, upon mixing of these renatured fragments, the

specific functional and structural properties of native thermolysin S are recovered. To this aim, further studies are needed to ascertain whether the individual chains of thermolysin S can be recombined in the presence of Ca^{2+} and Zn^{2+} ions to regenerate an active enzyme. Preliminary experiments on the conformational properties of fragment ThS-2 indeed indicated that the fragment is able to refold by itself to a native and stable structure, as judged by CD and immunochemical experiments (C. Vita et al., unpublished data). Thus, fragment ThS-2 appears to be an autonomous folding unit, although it corresponds to the COOH-terminal part of the original polypeptide chain, i.e., to that which is biosynthesized last.

In summary, the isolation and initial characterization of thermolysin S herewith described could provide a useful protein species for examining specific relationships between the structure and function of the enzyme. It will be of interest to examine the action of other proteases besides subtilisin for obtaining limited cleavage of thermolysin. As already found with other proteins (Neurath, 1980), it seems likely that different proteases will cleave thermolysin at the same exposed and flexible loops, but the actual site(s) of cleavage can vary, due to the fact that the specificities of the proteolytic enzymes employed would determine slight differences in the actual site of attack. In fact, preliminary experiments carried out in our laboratory of limited autolysis of thermolysin indicated that similar fragments to ThS-1 and ThS-2, as shown by SDS gel electrophoresis, can be obtained. Thus, limited proteolysis of thermolysin with various proteases could allow the isolation of nicked enzyme species slightly different from thermolysin S. These species will be useful to learn more about the structure and function of thermolysin.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Experimental details on the CNBr reaction of thermolysin S, a table reporting the analysis of the amino acids released by carboxypeptidase Y digestion of fragment ThS-2, two tables showing the amino acid composition of fragments 5–120 and 206–224(225), and four figures showing the elution profile from a Sephadex G-25 column of the CNBr reaction mixture of thermolysin S, the far- and near-ultraviolet CD spectra of thermolysin and thermolysin S, and the effect of pH and heat on the activity of thermolysin and thermolysin S (5 pages). Ordering information is given on any current masthead page.

Registry No. FAGLA, 26400-33-9; thermolysin S, 9073-78-3; subtilisin, 9014-01-1.

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High Mobility Group Like Chromosomal Proteins from Amoebas of the Acellular Slime Mold *Physarum polycephalum*†

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ABSTRACT: Amoebas of the slime mold *Physarum polycephalum* contain four major non-histone chromosomal proteins called AS-1, AS-2, AS-3, and AS-4. These proteins were extracted from chromatin with 0.35 M NaCl and were soluble in 2% trichloroacetic acid. Fractions AS-1, AS-2, and AS-3 were also quantitatively extracted with 5% perchloric acid, while a low yield of AS-4 was obtained with this method. Two minor non-histone proteins, AS-5 and AS-6, were extracted from nuclei, but not from chromatin, with either 5% perchloric acid or 0.35 M NaCl. These proteins had electrophoretic mobilities similar to HMG-18 and HMG-19 of calf. On acetic acid–urea–polyacrylamide gel electrophoresis, none of the four major *Physarum* AS proteins comigrated with the standard calf high mobility group (HMG) proteins. On sodium dodecyl sulfate gel electrophoresis, AS-1 migrated slower than calf HMG-1 and HMG-2 while AS-2 migrated near these two calf proteins. The AS-3 and AS-4 proteins, with apparent molecular weights of 16 500 and 16 000, respectively, migrated in the same region as calf HMG-14. The four major *Physarum* AS proteins were fractionated by exclusion chromatography on Bio-Gel P-100, and the amino acid compositions of the isolated proteins were determined. The four AS proteins contained high levels of both acidic and basic residues, a distinctive feature shared by most HMG proteins. Although some similarities in amino acid composition were seen between the *Physarum* and the calf HMG proteins, the results suggested that considerable divergence had occurred. A greater similarity was seen when the *Physarum*, *Tetrahymena*, and yeast HMG-like proteins were compared. The presence of HMG-like proteins in the slime mold *Physarum polycephalum* is further proof of the ubiquitous occurrence of the HMG proteins in eucaryotes.

It is only recently that histones from the acellular slime mold *Physarum polycephalum* were isolated and characterized. Using either plasmodia (Chahal et al., 1980; Mende et al., 1983; Champagne et al., 1982) or amoebas (Côté et al., 1982), two different developmental stages of this lower eucaryote, it was shown that *P. polycephalum* contained recognizable H1, H2A, H2B, H3, and H4 histones.

In addition to the histones, considerable attention has been focused on another group of chromosomal proteins, the high mobility group (HMG) proteins originally isolated from calf thymus nuclei (Goodwin et al., 1973). Although some of the HMG proteins appear clustered in the transcriptionally active regions of chromatin (Levy-Wilson et al., 1979; Weisbord et al., 1979), the precise function of these non-histone chromo-

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