The Proteolytic Enzymes of the K-1 Strain of *Streptomyces griseus* Obtained from a Commercial Preparation (Pronase)

IV. STRUCTURE-FUNCTION STUDIES OF THE TWO SMALLEST SERINE ENDOPEPTIDASES; STABILIZATION BY GLYCEROL DURING REACTION WITH ACETIC ANHYDRIDE*

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SUMMARY

A study was carried out on some of the properties of the two smallest serine endopeptidases from Pronase which had previously been purified to homogeneity. Each enzyme is homologous with bovine chymotrypsin and has isoleucine as the NH₂-terminal residue. The smaller enzyme is free of lysine, whereas the larger enzyme contains only 1 lysine residue. Reaction of the larger enzyme with acetic anhydride yielded a homogeneous, active, and stable derivative as indicated by ion exchange chromatography and acrylamide gel electrophoresis. Reaction of the smaller enzyme with acetic anhydride yielded two chromatographic components, of which only the larger demonstrated activity against N⁺-acetyl-L-tyrosine ethyl ester. This active component auto-
lizes during acrylamide gel electrophoresis but appears as a single band by cellulose acetate electrophoresis. The excellent yields of these protein derivatives were only achieved by modifying the past standard techniques of acetylation. In each reaction mixture glycerol was included at a concentration of 20% by volume. As a result only small amounts of native proteins were required to prepare the derivatives. Analysis of each enzyme revealed complete acetylation of the NH₂-terminal isoleucine residue. Acetylation resulted in only modest changes in the Michaelis constant and maximal velocity of each enzyme with N⁺-acetyl-L-tyrosine ethyl ester as substrate. Despite the earlier observation that only the larger enzyme demonstrated marked stability in 6 M guanidinium chloride there was no difference in the heat stabilities of the two enzymes. Neither showed an effect of ethylenediaminetetraacetate (EDTA) on activity at 37° even after several hours; however, at temperatures above 45° each enzyme underwent marked loss of activity in the presence of EDTA, whereas activity was conserved up to 60° in the absence of chelating agent. Metal-free enzyme was prepared in each case by gel filtration at a low pH in the absence of metals. These metal-free proteins demonstrated the same temperature stabilities as the EDTA-treated native enzymes. Of the many cations tested, Ca²⁺ was specific in each case in restoring the stability at higher temperatures to the metal-free enzymes.

In an earlier report the purification of four serine endopeptidases in Pronase was described (2). These enzymes have also been isolated to varying degrees of purity in other laboratories (3-6). The three smaller enzymes are homologous with mammalian chymotrypsin (2, 7, 8), whereas, in contrast, the largest enzyme demonstrated at least partial homology with the subtilisins (2). The two smallest enzymes have activity against N⁺-acetyl-L-tyrosine ethyl ester. The third enzyme of the chymotrypsin family of proteins hydrolyzes N⁺-benzoyl-L-arginine ethyl ester and, therefore, not unexpectedly demonstrated extensive homology with bovine trypsin (8). To date, all of the enzymes of the chymotrypsin family of serine proteases have either an isoleucine or valine residue which is implicated in a functional role at the active site (9). Because of the findings of an NH₂-terminal isoleucine in each of the two smallest serine enzymes in Pronase, the effect of extensive acetylation on the function of each protein was examined. A modification of the usual technique of the reaction of proteins with acetic anhydride was developed in order to obtain high yields of homogeneous derivatives from modest amounts of native precursors. The larger of the two enzymes was demonstrated earlier to conserve activity in the presence of either 6 M guanidinium chloride or 8 M urea (9). This enzyme demonstrated significant autolysis in 6 M guanidinium chloride if EDTA

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was present. In the absence of denaturant no effect of the chelating agent was found. Therefore, an investigation was carried out to discover the specific cation responsible for stabilizing the enzyme.

As will be demonstrated, the smaller of the two enzymes contains no lysine. Since both enzymes demonstrate elastase activity (10) and also, like chymotrypsin, hydrolyze Ac-Tyr-OEt, we propose the trivial names lysine-free chymoelastase and guanidine-stable chymoelastase for the smaller and larger proteins, respectively.

MATERIALS AND METHODS

Pronase (grade B) was obtained from Calbiochem; several lots (numbers 900053, 000130, 000133, and 001828) were used for these studies. The serine proteases were purified as previously described (1). Only the two smallest enzymes with activity against Ac-Tyr-OEt were utilized in these studies. They were determined to be homogeneous by chromatography through CM-cellulose, by gel filtration, and by acrylamide gel electrophoresis. Ac-Tyr-OEt and Na-acetyl-L-phenylalanine ethyl ester were obtained from Mann Research Laboratories. Casein was obtained from Difco and purified (1). Urea, reagent grade, was obtained from Mallinckrodt, and guanidinium HCl of highest purity was obtained from Eastman Kodak. These denaturants were purified further as described earlier (8). Preswollen microgranular CM-cellulose (Whatman CM-52) was obtained from Reeve Angel. Sephadex G-25 was obtained from Pharmacia Fine Chemicals. Polyamide layers, a product of the Chung Chin Trading Co., Ltd., were purchased from Gallard-Schleisinger. The sheets (15 x 15 cm) were quartered before utilization for analysis of dansyl amino acid derivatives. All other chemicals were of reagent grade.

Amino Acid Analyses—Analyses were performed according to standard techniques (11) on a Beckman amino acid analyzer. Acid hydrolysates (24-, 48-, 72-, and 96-hour) with about 2 mg of protein per tube were carried out in vacuo with 6 N HCl at 105°C. Cysteine and methionine were analyzed as their oxidized products following reaction with performic acid (12). Reported serine and threonine values are those obtained after extrapolation to zero time of hydrolysis (13). Tryptophan content was determined by a spectrophotometric assay in base (14). The quantitation of NH2-terminal residues was by the cyanate procedure (15) after the proteins had been oxidized by performic acid (12). Chromatography through a Dowex 1-X8 column was carried out in order to remove peptides containing cysteic acid from the hydantoins (15). The recoveries for the NH2-terminal residues were calculated using the data of Stark (15) without accounting for losses after passage through the Dowex 1 column. Therefore, the values reported here are probably low.

Measurement of Catalytic Activity—The extent of casein digestion was determined as described earlier (9). The activities against Ac-Tyr-OEt and Ac-Phe-OEt were measured by previously described techniques using the pH stat (16). The kinetic constants toward Ac-Tyr-OEt and Ac-Phe-OEt were determined at substrate concentrations from 5 to 30 mM. The enzyme concentrations for each assay are listed in Table I. The assay medium in the kinetic studies contained 3% dioxan to permit the complete solubility of the ester substrates at the higher concentrations. Titrations were performed at pH 8.0 at 25°C with 0.049 M NaOH. The data was interpreted according to the method of Lineweaver and Burk (17). The pH dependence of activity against Ac-Tyr-OEt and Ac-Phe-OEt was studied as previously described (1).

Acetylation of Proteins—the technique of Oppenheimer et al. was followed in reacting each enzyme with acetic anhydride (18). However, because of the paucity of material the procedure was scaled down considerably. Five or 10 mg of enzyme were added to 3 ml of 5 mM sodium acetate-5 mM calcium acetate (pH 5.0). The solution was cooled to 2°C in a water-jacketed vessel. The pH was raised to 6.7 and maintained at this level by the addition of 2 M NaOH with the use of a pH-stat (Radiometer TTT1). Constant stirring was maintained with a magnetic stirrer. Acetic anhydride was added (5 μl every 7 min) for six or seven additions. Following the final addition the reaction was allowed to proceed for a further 30 min. Despite the great care taken in the slow addition of the anhydride, substantial amounts of protein precipitated irreversibly from solution. The remaining protein in solution was polydisperse when analyzed by CM-cellulose chromatography and, furthermore, was inactive when tested against Ac-Tyr-OEt. Attempts were made without success to circumvent this problem by running the reaction in half-saturated sodium acetate in order to reduce the number of acetylated tyrosine residues (19). Halting the reaction with anhydride at the inception of precipitate formation or running the reaction in 4 M urea was unsatisfactory. These latter methods yielded heterogeneous products with incomplete reaction and little activity. The problem of gross denaturation was eliminated when the standard reaction was carried out with the addition of glycerol (20% by volume). With this modification no precipitate was noted. An entirely satisfactory product was obtained in the case of the guanidine-stable chymoelastase, but in the case of the lysine-free chymoelastase two chromatographic components were noted (see below). Acetylation of the latter enzyme in the presence of 13 or 30% glycerol (by volume) yielded results less satisfactory than those achieved at 20% concentration.

The native proteins and acetylated derivatives were separately analyzed by chromatography through a CM-cellulose column (1.1 x 7.5 cm) in the sodium and calcium acetate buffer as previously described (2). Because of the small amount of protein applied, the effluent fractions were monitored for absorbance at 230 nm. The glycerol in the solutions containing the acetylated proteins was removed by passage through a Sephadex G-25 column equilibrated with the above buffer before the proteins were passed through the ion exchange column.

The homogeneity of native and acetylated guanidine-stable chymoelastase was analyzed by electrophoresis in polyacrylamide gel at pH 4.3 as described earlier (2). Twenty microliters of protein solution were added for each run. The amount of protein applied was determined according to the method of Lowry et al. (20) with bovine serum albumin used as the standard. The gels were stained with Amido Schwarz. Acetylated lysine-free chymoelastase was not stable in this system. Therefore, this derivative and its precursor were analyzed by electrophoresis on cellulose acetate membranes with a Beckman Microzone system. The buffer composition (sodium diethylbarbiturate, pH 8.6) and staining technique with Ponceau S were those described by the Beckman Methods Manual (RM-TB-010).

The NH2-terminal residues of the native and acetylated proteins were examined qualitatively after performic acid oxidation by reaction with dansyl chloride (21). The results were analyzed by two-dimensional chromatography on quartered polyamide sheets (22).
Table I

Amino acid compositions

| Amino acid residue | Lysine-free chymoelastase | Guanidine-stable chymoelastase |
|--------------------|---------------------------|-------------------------------|
|                    | moles/15,700 g protein    | moles/18,000 g protein        |
| Lysine             | 0                         | 1.02                          |
| Histidine          | 2.86                      | 1.97                          |
| Arginine           | 5.82                      | 7.85                          |
| Aspartic acid      | 13.70                     | 36.40                         |
| Threonine          | 18                        | 27                            |
| Serine             | 19                        | 20                            |
| Glutamic acid      | 7.35                      | 4.94                          |
| Proline            | 3.59                      | 4.91                          |
| Glycine            | 26.48                     | 32.25                         |
| Alanine            | 18.50                     | 14.87                         |
| Half-cystine       | 2.66                      | 2.96                          |
| Valine             | 11.10                     | 14.23                         |
| Methionine         | 0.50                      | 1.52                          |
| Isoleucine         | 7.02                      | 6.80                          |
| Leucine            | 8.76                      | 6.95                          |
| Tyrosine           | 6.25                      | 8.96                          |
| Phenylalanine      | 4.48                      | 5.08                          |
| Tryptophan         | 1.7                       | 3.3                           |

* Entries are values extrapolated to zero hydrolysis time.
* Determined as cysteic acid.
* Entries are maximum amounts of amino acids released after 72 hours of hydrolysis.
* Determined as methionine sulfoxide.
* Estimated by spectrophotometric analysis (14).

Spectra of native and acetylated proteins in 5 mM sodium acetate-5 mM calcium acetate (pH 5.0) were measured with a recording spectrophotometer (Zeiss DMR 21).

Heat Stability and Calcium Dependence—The heat stability of each enzyme was studied in the presence and absence of EDTA. One milligram of each enzyme was dissolved separately in 0.5 ml of 10 mM Tris (pH 8.0). Thereafter 25 µl aliquots were added to 75 µl of 10 mM Tris (pH 8.0) containing either 10 mM EDTA or 10 mM CaCl₂. The solutions were incubated at the desired temperature for 10 min and then plunged into ice. After 1 hour at 0º, a 50-µl aliquot was used to assay for activity against Ac-Tyr-OEt. All results have been expressed as percentages of the maximal activity noted.

The following procedure was carried out to determine the specific cation which stabilized the two enzymes against heat denaturation. Five milligrams of each enzyme were dissolved separately in 1.0 ml of 0.1 M glycine (pH 3.4) containing 10 mM EDTA. It was later appreciated that at this pH EDTA has a very low affinity for divalent cations and therefore probably contributed nothing to these studies (23). The solution was passed through a Sephadex G-25 column equilibrated with the above glycine solution. The fractions containing the eluted protein were combined (total volume was 6 ml). Fifty-micro-liter aliquots were added to separate tubes containing 50 µl of different chloride salts at 0.1 M concentration. Immediately thereafter 300 µl of 0.1 M Tris buffer (pH 8.0) were added. The aliquots were incubated for 10 min at the desired temperature. After cooling for 1 hour at 0º, 100-µl aliquots were removed to assay for activity against Ac-Tyr-OEt. These results were expressed as percentages of the activity noted after incubation in 12.5 mM CaCl₂ and the above Tris buffer at 24º.

Stability of Lysine-free Chymoelastase in Denaturant—The stability of lysine-free chymoelastase in either urea or guanidinium HCl was determined in the presence of either CaCl₂ or EDTA. Solutions of 0.1 M Tris-HCl (pH 8.0) were made containing 3 M guanidinium HCl, 6 M guanidinium HCl, or 8 M urea with either 10 mM CaCl₂ or 1 mM EDTA. To 1.0-ml aliquots of each of these solutions was added 0.5 mg of enzyme in 50 µl of distilled H₂O. After incubation at room temperature for various time periods 100-µl aliquots were removed for assay against Ac-Tyr-OEt (1, 2, 16).
protein, the NH2-terminal isoleucine. Acetylation of this amino group and of tyrosine residues may be hindering the interaction of arginine residues with the CM-cellulose (see below).

The homogeneity of acetylated guanidine-stable chymoelastase as demonstrated by acrylamide gel electrophoresis is depicted in Fig. 3. The derivative, as expected, migrates less rapidly toward the cathode than the parent protein. The acetylated protein demonstrated the poor retention of Amido Schwarz as noted earlier with the native enzyme (2, 9). The derivative, like the native enzyme, showed stability in 6 M guanidinium HCl. Therefore, the loss of dye was attributed to diffusion of the stable enzyme-dye complex from the gel (9). Acetylated lysine-free chymoelastase was not stable in the conditions of gel electrophoresis; no stainable material was discerned after the run. This derivative was reacted with diisopropylphosphorofluoridate with apparent complete inhibition of activity. Electrophoresis of the double derivative through acrylamide gel revealed the presence of several bands. The possibility was considered that the heterogeneity arose from denaturation during acrylamide gel electrophoresis; therefore, acetylated lysine-free chymoelastase was examined by cellulose acetate membrane electrophoresis. Fig. 4 gives the results of studies on native and acetylated enzyme after reaction with diisopropylphosphorofluoridate; it appears by this technique that the proteins are homogeneous. Furthermore, as expected from the absence of lysine in this protein, there is little difference in the migration of acetylated and native proteins.

Fig. 5 demonstrates the results of studies on the NH2-terminal residues of the native and derivative proteins. Guanidine-stable chymoelastase has only isoleucine as the NH2-terminal residue. Lysine-free chymoelastase has primarily isoleucine, as noted by the very large fluorescent spot of dansyl-isoleucine; however, this enzyme also demonstrates several other dansyl residues in very low amounts. These other NH2-terminal residues are probably generated during the reaction with dansyl chloride, since the parent native enzyme was shown to be homogeneous by acrylamide gel electrophoresis (2). The acetylated proteins demonstrate the absence of patterns of any dansyl amino groups, indicating that the amino groups have been completely acetylated.
FIG. 5. Dansyl amino acid patterns obtained from the acid hydrolysates of Pronase enzymes that were treated with dansyl chloride. Top panels, guanidine-stable enzyme; bottom panels, lysine-free enzyme; left panels, native enzymes; right panels, acetylated enzymes. Material derived from about 2 nmoles of protein was applied in each panel. Positions of application of samples can be discerned by the dark spot or circle in the lower right quadrant of each panel. Migration in the water and formic acid solution from right to left and in the benzene and acetic acid solution from bottom to top (22). Photographs of fluorescence during exposure to an ultraviolet lamp (H384JM mercury flood lamp with Kopp filter No. 41 UV, Black Light Eastern Corp., New York, N.Y.).

FIG. 6. Ultraviolet spectra of native (—) and acetylated (— — —) proteins. A, lysine-free protease, 0.80 mg per ml of native or about 0.7 mg per ml of acetylated enzyme. B, guanidine-stable protease, 0.72 mg per ml of native or about 0.6 mg per ml of acetylated enzyme.

Fig. 6 demonstrates the spectra of native and acetylated proteins. The spectral shifts of each protein after acetylation may be due in part to O-acetylation of tyrosine residues. In contrast to the studies with chymotrypsin, the spectra of the derivatives were not converted back toward those of the native proteins after reaction with acetic at pH 5.0 for several days at 4° or by reaction with 0.1 M hydroxylamine for several hours at room temperature. If the spectral shifts are attributable to O-acetylation of tyrosine, these liganded groups appear to be unusually resistant to cleavage in these proteins.

FIG. 7. The effect of pH on the activity against Ac-Phe-OEt of native (●) and acetylated (○) enzymes. A, guanidine-stable chymoelastase; B, lysine-free chymoelastase.

Fig. 7 depicts the activities of guanidine-stable and lysine-free chymoelastases against Ac-Phe-OEt as a function of pH. The pH range of maximal activity lies between 7.5 and 10; no differences are seen between each native enzyme and its acetylated derivative.

Recently we described the unique stability in denaturant of guanidine-stable chymoelastase (9, 24). A study was carried out to examine the stability of lysine-free chymoelastase in urea and guanidinium HCl. Fig. 8 depicts the results. In the presence of 10 mM CaCl₂ this enzyme appears to be entirely stable in 7.6 M urea but loses activity in high concentrations of guanidinium HCl. In contrast, in 1 M sodium EDTA a moderately rapid loss of activity is noted in 7.6 M urea and the rate of inactivation in guanidinium HCl is accelerated. As previously noted with guanidine-stable chymoelastase (9), no effect of EDTA on the stability of lysine-free chymoelastase could be demonstrated in the absence of denaturant.

Table III demonstrates the kinetic constants of each enzyme under different conditions. The V_{max} of each enzyme toward Ac-Tyr-OEt is much less than that of bovine chymotrypsin (16). The K_{m} for Ac-Tyr-OEt of each enzyme increases greatly in 8 M urea. The acetylated enzymes show only slight differences in kinetic constants from those seen with the native proteins.

The studies with EDTA suggested that in each enzyme there were tightly bound metal ions which were only accessible to the
glycerol could restrict the degree of initial relaxation of conformationally flexible sites. The proteins could undergo further conformational transitions which would make them susceptible to autolysis. If buried groups susceptible to acetylation. When acetylated at higher temperatures the protective effect of a metal ion is no effect of chelating agent is demonstrable below 45°C; however, at higher temperatures the protective effect of a metal ion is inferred by the destabilizing effect of EDTA. There is no significant difference in the heat stabilities of the two enzymes. As described above, apparent metal-free enzyme was prepared in each case and an analysis was carried out on the protective effect of various cations on the heat stability at 55°C. As is demonstrated in Table IV, calcium was specific as the required metal for the two proteases.

### DISCUSSION

Glycerol has been used in many enzymological studies as an empirical measure to stabilize proteins. For instance, placental 17β-hydroxysteroid dehydrogenase when stored in a buffer with 50% glycerol was completely protected against loss of activity (25), in contrast to the rapid denaturation noted in glycerol-free solutions. At concentrations less than 50%, glycerol gave intermediate degrees of protection. Furthermore, glycerol protected the enzyme against heat denaturation. In another study, buffers with 50% glycerol completely protected aminoacyl ribonucleic acid synthetases against loss of activity when stored at -18°C (26). How glycerol stabilizes proteins remains obscure; to our knowledge no definitive studies have been carried out to determine the mechanism. The enzymes in the present study, in contrast to the above examples, are among the most stable soluble proteins known. Therefore, the addition of glycerol during our experiments requires some explanation. We considered that there might be substantial flexibility of the proteins despite their stability. This flexibility could permit the fleeting exposure of buried groups susceptible to acetylation. When acetylated at these sites the proteins could undergo further conformational transitions which would make them susceptible to autolysis. If glycerol could restrict the degree of initial relaxation of conformational changes in the presence of denaturant, the susceptible buried groups would not be acetylated. It is clear, in the case of guanidine-stable chymoelastase, that the major stabilizing effect of glycerol cannot be the restriction of conformational changes after acetylation. Purification of the acetylated derivative free of glycerol resulted in a completely stable protease. However, in the case of lysine-free chymoelastase the stabilization of the acetylated derivative by glycerol may also be important in view of the moderate rate of loss of activity following the removal of glycerol. Another explanation for these results may be that glycerol, as a competitive nucleophile, significantly improves the selectivity of acetylation. The reaction of glutamate dehydrogenase with acetic anhydride demonstrated significant improvement in selectivity when in the presence of 0.1 M Tris acetate, a nucleophilic buffer (27). Our very preliminary studies suggest the possibility that glycerol may generally stabilize protein substrates during other methods of chemical modification. Recently the carboxymethylation of sulfhydryl groups on aldehyde dehydrogenase was shown to be affected by concentrations of glycerol which stabilize that enzyme (28). Our procedure represents a significant advance over the methods previously used for acetylation of trypsin and chymotrypsin. By minimizing denaturation and heterogeneity of products we were able to effect a 100-fold reduction in scale.
of the acetylation procedure as compared with the studies of chymotrypsin and trypsin (18, 29).

The acetylation studies were carried out because of the identity of residues in the microbial enzymes Asp-194 and Leu-16 in α-chymotrypsin (2, 7, 9, 16). In the bovine enzyme these 2 residues form an ion pair via the β-carboxyl group of the aspartyl residue and the α-amino group of the terminal isoleucine (30). It has been demonstrated that specific substrates will bind to the enzyme only when this ion pair is formed (31). In α-chymotrypsin, acetylation of this α-amino group results in complete loss of activity towards specific substrates (18) but not towards p-nitrophenyl acetate (32). On the other hand, acetylation of the α-amino group of a homologous residue in porcine elastase results in no loss of activity (33). Finally, reaction of acetic anhydride with trypsin fails to acetylate the NH₂-terminal isoleucine (44-46). These results have been interpreted as providing the amino acid analyses. We are appreciative of the expert help of Mrs. Tina Siegel in the preparation of the figures.

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Fig. 10. Conversion of the ion-pair bond in the chymotrypsin family of serine proteinases to a postulated hydrogen bond after acetylation.
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