The Proteolytic Enzymes of the K-1 Strain of \textit{Streptomyces griseus} Obtained from a Commercial Preparation (Pronase)

II. THE ACTIVITY OF A SERINE ENZYME IN 6 M GUANIDINIUM CHLORIDE*

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SUMMARY

A homogeneous serine protease, homologous with bovine chymotrypsin, purified from a commercial preparation (Pronase) of the K-1 strain of \textit{Streptomyces griseus} demonstrated marked stability in the presence of 6 M guanidinium chloride. In this solvent the enzyme was active against N-$\alpha$-acetyl-L-tyrosine ethyl ester showing a slight increase in $K_m$ but a significant decrease in $V_{\max}$ as compared to results of studies in the absence of denaturant. The rate of hydrolysis of casein was enhanced in the presence of 6 M guanidinium chloride. Ovalbumin, which was minimally hydrolyzed in the absence of denaturant was extensively hydrolyzed by the enzyme in the presence of denaturant. These results probably reflect the selective unfolding of the substrate proteins by denaturant. The enzyme revealed no difference in circular dichroism spectra in the presence or absence of 6 M guanidinium chloride. Sodium ethylenediaminetetraacetate in the presence of denaturant caused a sustained irreversible loss of activity associated with a change in the circular dichroism spectrum. In the absence of guanidine, minimal effects of the chelating agent were seen. These results suggest the probable stabilizing effect of a cation. We believe this is the first demonstration of conservation of enzymatic activity in the presence of 6 M guanidinium chloride. This protease may be useful for structural studies of polypeptides which are, in the native state, resistant to proteolysis.

A companion report describes the facilitated purification to homogeneity of serine proteases present in a commercial preparation, Pronase, obtained from the K-1 strain of \textit{Streptomyces griseus} (1). Three of these enzymes are homologous with the mammalian pancreatic endopeptidases. Varying degrees of purification of these enzymes have been reported by several other laboratories (2–6). In the course of studying some of the properties of that enzyme with the greatest affinity for carboxymethylcellulose, the stability of the enzyme in the presence of some denaturants was analyzed. This enzyme with an estimated molecular weight of 17,500 is also an esterase with activity against N-$\alpha$-acetyl-L-tyrosine ethyl ester. It had been noted during the course of analytical studies for purity by acrylamide gel electrophoresis at pH 4.3 that this protease demonstrated curious staining properties. Treatment of the gels with either Amido schwarz or Coomassie blue gave the usual coloration of a single protein band. However, after a period of several days following the application of Amido schwarz or 2 to 3 weeks following the application of Coomassie blue, each gel was almost completely decolorized. This occurred in an acidic solution where the enzyme is completely inactive. Restaining yielded no further dye retention. This suggested that the protease had diffused from the gel. A general inquiry revealed that this characteristic had not been previously observed. Therefore, the consideration arose that this enzyme might be unusually resistant to denaturation.

MATERIALS AND METHODS

Pronase (grade B) was obtained from Calbiochem; several lots (numbers 900053, 000130, and 000333) were used for these studies. The serine proteases were purified as previously described (1). Pronase, obtained from the K-1 strain of \textit{Streptomyces griseus} (1). Three of these enzymes are homologous with the

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chloride of highest purity was obtained from Eastman Kodak. It was further purified by dissolving in boiling methanol (1.0 g in 1.5 ml). After cooling the solution in an ice bath, 14 volumes of cold diethyl ether was added (7). The filtered precipitate was dried under vacuum in a desiccator.

The activity against Ac-Tyr-OEt was determined by previously described techniques (8). The kinetic constants toward Ac-Tyr-OEt were determined at substrate concentrations of 5 mM to 30 mM and enzyme concentrations of 0.015 to 0.03 mg per ml. The assay medium in the kinetic studies contained 3% dioxane to permit the complete solubility of the ester substrate at the higher concentrations. Titrations were performed at pH 8.0 at 25° with 0.049 M NaOH. The extent of casein or ovalbumin hydrolysis by the enzyme was determined after incubation at 37° by measuring the amount of supernatant absorbance at 280 nm after precipitation with trichloroacetic acid. Protein substrates in guanidine stood at room temperature for 1 hour to allow time for unfolding before the addition of the protease. Aliquots (1 ml each) of incubation mixtures were utilized for each assay. In order to reduce the differential effect of guanidinium chloride on the assays, the following procedure was done. To those aliquots without guanidine, 1 ml of 6 M denaturant was added; to those with guanidine, 1 ml of water was added. Immediately thereafter 2 ml of 10% trichloroacetic acid was added to each sample. Each experiment was terminated when no further increase in absorbance at 280 nm was noted or when no material could be precipitated after addition of the trichloroacetic acid solution. In the latter case the optical densities of the soluble peptides gave values close to those expected for the complete solubilization of the parent proteins.

Measurements of circular dichroism were performed on a Cary 60 spectropolarimeter with CD attachment. The instrument was standardized with an aqueous solution of d-10-camphorsulfonic acid (K and K Laboratories, Plainview, N.Y., batch 4829), giving [θ] = 7260 ± 165 deg cm2 per decimole at 290 nm. Spectra were obtained on solutions in the protein concentration range of 1 to 2 mg per ml at 25°. Determinations of protein concentration were made from the absorbance for which the correspondence is ε280° = 8.1 at 280 nm. This value was determined by the standard technique with the Folin phenol reagent with bovine serum albumin as a standard (9). Concentrated enzyme samples were first prepared in 4 mM Tris (pH 8.0) containing 4 mM CaCl2. Solutions of guanidinium chloride (6.3 M) or urea (8.4 M) in the same buffer were added to separate aliquots of protein solution for a final guanidine concentration of 6.0 M and a final urea concentration of 8.0 M.

RESULTS

Fig. 1 demonstrates the results of a study of the stability of the enzyme in 6 M guanidinium chloride. The incubations were carried out at the pH of maximal activity for this enzyme. Retention of activity was noted and increasing calcium ion concentrations appeared to protect against slight loss of esterase activity. Therefore, these results could mean either the unusual retention of the native state in this denaturant or refolding of completely denatured enzyme upon dilution in the guanidine-free assay medium. The significant finding was the effect of sodium EDTA. With excess chelating agent there appeared a moderately rapid loss of activity. The loss of activity was not reversible if excess calcium chloride was added to an aliquot with a 1-hour incubation before assays against Ac-Tyr-OEt; nor was the activity restored when an aliquot was diluted into a guanidine-free solution containing excess calcium ion at pH 8.0. These findings suggested that in the absence of EDTA the enzyme retained conformational stability in the denaturant. It was assumed without further evidence that calcium ion is the only cation involved in the stabilization process. Very slight inhibition of this enzyme by EDTA was noted in the absence of denaturant. A separate experiment was carried out under conditions similar to that listed in Fig. 1 to determine the long term stability of the Pronase enzyme in denaturant. The incubation solutions included 4 mM CaCl2 in the absence of chelating agent.

![Fig. 1 (left). Stability of Pronase enzyme in 6 M guanidinium chloride. Enzyme concentration was 0.08 mg per ml in 0.1 M Tris (pH 8.0) with denaturant incubated at 25° in 0.01 M CaCl2 (O—O), 0.001 M CaCl2 (△—△), 0.0001 M CaCl2 (Δ—Δ), or 0.001 M sodium EDTA (■—■). Aliquots (200 μl each) were removed for rate assays of hydrolysis of Ac-Tyr-OEt; the only denaturant in each assay was the amount transferred in the enzyme aliquot. The total volume in each assay was 0.2 ml.](http://www.jbc.org/)

![Fig. 2 (center). Hydrolysis of casein by Pronase enzyme in the presence and absence of 6 M guanidinium chloride with guanidine (△—△) or without guanidine (O—O). Conditions were: casein concentration of 0.5 mg per ml, enzyme concentration of 0.07 mg per ml, in 0.047 M Tris (pH 8.0), 0.047 M KCl, and 0.0047 M CaCl2, incubation at 37°.](http://www.jbc.org/)

![Fig. 3 (right). Hydrolysis of ovalbumin by Pronase enzyme in the presence and absence of 6 M guanidinium chloride with guanidine (△—△) or without guanidine (O—O). Conditions were: ovalbumin concentration of 9.5 mg per ml, enzyme concentration of 0.07 mg per ml, in 0.1 M Tris (pH 8.0), 0.1 M KCl, and 0.01 M CaCl2, incubation at 37°.](http://www.jbc.org/)
After 20 hours the enzyme in 6 M guanidine contained 75% and the enzyme in 8 M urea, 82% of the activity of a control solution without denaturant. The activity of the enzyme against Ac-Tyr-OEt was studied in the presence and absence of 6 M guanidine chloride, without dilution. Table I shows that the denaturant causes a slight increase in $K_m$ but a marked decrease in maximal velocity. Thus the enzyme is definitely active in the denaturant. The decrease in maximal velocity probably represents diminished activity of most of the enzyme molecules rather than maximal activity of a small fraction of the molecules, because maximal activity returned after dilution of the enzyme into guanidine-free solutions. It would be unlikely that this represented extensive reversible renaturation of most of the molecules for one would expect untold species to be susceptible to rapid autolysis.

Fig. 2 demonstrates the effect of the enzyme on casein in 6 M guanidinium chloride. The rate of proteolysis is apparently greater in the presence than in the absence of denaturant. No difference is noted in the extent of proteolysis. This rate enhancement is probably not due to a general salt effect. In the presence of 2 M KCl the casein solution became more opaque during the reaction with the Pronase enzyme and a diminished apparent rate of hydrolysis was noted. If the velocity of peptide bond cleavage was reduced in 6 M guanidine to a degree similar to the reduction of esterase rate, it follows that the increase in rate of hydrolysis must reflect a much greater accessibility of substrate to enzyme in denaturant. A similar effect of guanidine on the hydrolysis of casein by streptococcal proteinase has been reported (10).

Fig. 3 demonstrates the effect of the enzyme on ovalbumin. By 2 hours the extent of apparent proteolysis in denaturant is 35 times greater than that achieved in the absence of guanidine. In fact minimal apparent digestion is seen without denaturant. Crude Pronase, a mixture of many proteases, hydrolyzes native ovalbumin extensively (11), an observation differing from that of the present study with the purified single component. In contrast to the study with casein, a protein considered to be randomly coiled in the absence of denaturant, the proteolysis of ovalbumin necessarily first required the unfolding of substrate. Fig. 4 depicts the circular dichroism of native and denatured enzyme. In Tris buffer, 8 M urea or 6 M guanidinium chloride solution the spectrum of the native enzyme is unchanged. This supports the view that the diminished esterase activity in guanidine does not reflect gross denaturation of a large fraction of the enzyme molecules. However, undetected minor conformational changes could account for the difference in the activity against Ac-Tyr-OEt. Enzyme activity decreases over a 3-hour period when EDTA is added to the guanidinium chloride solution. This loss of enzyme activity corresponds in part to changes seen in the CD spectrum with the 215 nm minimum becoming less pronounced by 10 to 15%. However, CD spectral changes continue to occur subsequent to the diminution in enzyme activity. Over

### Table I

| Condition                        | $K_m$ (m) | $V_{max}$ (sec⁻¹) |
|----------------------------------|-----------|------------------|
| Without guanidine…               | 0.024     | 14               |
| With 6 M guanidinium chloride…  | 0.034     | 2                |

Fig. 5 demonstrates the stability of Pronase enzyme in 8 M guanidinium chloride. Enzyme concentration was 0.38 mg per ml in denaturant with 0.006 M Tris (pH 8.1), 0.01 M CaCl₂, and 0.005 M KCl incubated at 37°. Aliquots (40 μl each) were removed for rate assays of hydrolysis of Ac-Tyr-OEt; the only denaturant in each assay was the amount transferred in the enzyme aliquot. The total volume in each assay was 3.04 ml.
The results of this investigation demonstrate conformational stability with retention of activity of an enzyme in 6 M guanidinium chloride. This stability is presumably dependent upon some cation since sodium EDTA causes moderately rapid irreversible denaturation. It has been suggested that 6 to 8 M guanidinium chloride may not be sufficient to effect a conformational change for exceptionally stable proteins (12). This consideration is apparently borne out by the present example. It has been noted that block copolymers containing either poly- oleucine or polyphenylalanine retain helical stability in 7.2 M guanidine (13). In the present study, the CD curve of the native enzyme demonstrates very little evidence of helical structure. The shoulder at 230 nm is not particularly sensitive to denaturing conditions, a feature suggesting that this band belongs primarily to a nonhelical contribution to the optical activity. Therefore, other structural features must be considered to explain the present example of stability.

The following meager facts are known about the structure of this protein. Like bovine chymotrypsin there is the Asp-Ser-Gly sequence around the reactive serine residue (14). Recent studies in this laboratory have demonstrated isoleucine to be the only amino-terminal residue. Amino-terminal isoleucine or valine is found in the members of the chymotrypsin family and has been implicated in their conformational stability (15). Studies on the sequence of the trypsin contained in Pronase have demonstrated significant homology with bovine trypsin (16). It would appear likely that the Pronase component under present investigation would demonstrate a similar degree of homology. The amino acid composition reveals 12 arginine and 2 lysine residues per molecule. Five to 6 half-cystine residues are found and since preliminary studies reveal no free sulphydryl groups, these are probably fully incorporated into disulfide bonds. There could not be a very large number of these bonds to account for the marked stability.

Several studies have been done on the stability of chymotrypsin in urea and guanidinium chloride (17-19). It was demonstrated that the bovine enzyme was stable at low concentrations of either denaturant. However, in 8 M urea or at intermediate concentrations of guanidinium chloride, there was rapid denaturation. The denaturation was irreversible if carried out at pH values in the enzyme activity range. At guanidine concentrations of 5 M or greater the denaturation was essentially reversible. The partial stabilization of conformation by calcium ion was also demonstrated. The conclusions were that the enzyme at a certain concentration of denaturant was in an equilibrium between native and unfolded conformations. Therefore, at pH values in the enzyme activity range rapid autolysis occurred. At high guanidine concentrations essentially only unfolded species of protein existed and thus autolysis was insignificant. In contrast, the present study in 6 M guanidinium chloride of the related S. griseus enzyme demonstrates retention of protease and esterase activities without great change in conformation. This finding reflects the tendency of microbial proteases to be more stable than the pancreatic proteases as exemplified in the studies with subtilisin (20).

Some of the proteolytic activities in crude Pronase appear to be conserved in 8 M urea (21). However, the activity in denaturant against Ac Tyr OEt was not reported. Studies in this laboratory have shown that neither of the two other components which are homologous with chymotrypsin nor the component which is homologous with subtilisin (1) is stable in 6 M guanidine. Commercial Pronase contains a significant amount of undefined low molecular weight material. The stable component described in the present report represents only about 15% of the protein content. Therefore, at least partial purification of the stable component would be required for definitive studies of the hydrolysis of substrates in 6 M guanidine.

The stabilization of several of the components in Pronase by calcium ion was recognized very early (22). At 4°C with excess EDTA irreversible loss of 70% of the protease activity was noted within 10 min. Thereafter, over a period of 3 hours, no further loss of activity was detected. Dialysis against various cations revealed that only calcium ion prevented significant loss of activity. A later study carried out after the recognition of the multicompontent nature of Pronase showed no inhibition by EDTA of chromatographic fractions containing the enzyme under present investigation (2). The present study confirms this finding; for, it is only with denaturant that a significant inhibition by EDTA is detected. This effect of EDTA in denaturant suggests that the enzyme is structurally less rigid in denaturant but that the susceptibility to proteolysis is only slightly increased. If autolysis is primarily an intermolecular event this differential effect between chelating agent and another protease molecule would not be surprising. For, a small molecule such as EDTA in contrast to a protein molecule might easily be accommodated by the interstices of a slightly unfolded enzyme. It is to be noted that calcium ion was present at a concentration of at least 5 mM at every step of purification of this protease.

Preliminary studies in this laboratory of the Pronase enzyme have not revealed marked thermal stability. The property of significant retention of the native state of this enzyme in urea or guanidine may be of use in the structural studies of some polypeptides. Certain problems may arise with the standard procedures for the hydrolysis of proteins by specific endopeptidases. Initial preparation of the substrate usually includes some form of denaturation since native proteins are generally resistant to proteolysis. These techniques for irreversible denaturation may result in the cleavage of certain covalent bonds such as the oxidation of disulfide bonds. In addition, proteolysis of the denatured protein in many cases is incomplete leaving an insoluble residue. The use of this enzyme with denaturant should in principle by-pass these two complications. The specificity of this protease is under investigation to determine its applicability in sequence studies. An important consideration will be the determination of conservation of specificity in denaturant.

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