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

STABILIZATION OF THE TRYPsin COMPONENT BY CALCIUM AND GUANIDINE* 

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SUMMARY

*Streptomyces griseus* trypsin is more thermolabile than the two other components in pronase which are homologous with bovine chymotrypsin. It is completely inactivated after heating to 60° for 15 min. The heat stability of the enzyme is reduced in the presence of EDTA. Calcium was found to be the specific cation which stabilizes the enzyme at higher temperatures. This trypsin denatures irreversibly in 8 M urea (at 23°) in low calcium ion concentrations, but is stable and active in this denaturant if 0.5 M calcium ion is present. This latter property makes this enzyme a possibly useful agent in protein structural studies.

Both the microbial and bovine trypsin are found to bind guanidinium ion substantially. Guanidinium ion competitively inhibits the activity of each enzyme against N°-benzoyl-L-arginine-p-nitroanilide. Microbial trypsin has about a 3-fold greater affinity for guanidine and about a 20-fold lower K_m for N°-benzoyl-L-arginine-p-nitroanilide than does bovine trypsin. Binding of guanidine with either enzyme produces no apparent inhibition of activity against the poor nonspecific substrate, p-nitrophenyl acetate, when compared to inhibitor-free solutions. These findings suggest that guanidine associates with that part of the specificity site which binds the charged portion of basic substrate residues.

The addition of 0.2 M guanidine HCl to an 8 M urea-10 mM CaCl_2_ solution completely inhibits the autolysis of the microbial trypsin but only slightly decreases the rate of autolysis of the bovine enzyme. In 8 M urea-10 mM CaCl_2_ and 1.0 M guanidine HCl, about 90% of the activity of the microbial enzyme is retained after 2 hours even in the presence of another *S. griseus* serine endopeptidase known to be active and stable in this mixed denaturant solution. Therefore, guanidine appears to stabilize microbial trypsin. In the presence of sodium ethylenediaminetetraacetate and denaturant mixture, the microbial enzyme rapidly loses activity.

Measurements of circular dichroism were made at pH 8 and revealed that 0.45 M CaCl_2_ completely protects the microbial enzyme against rapid unfolding by 8 M urea, whereas this salt has little effect upon the rapid conformational transition of the bovine enzyme in this denaturant. It was found also that 0.2 M guanidine with a low calcium ion concentration can largely stabilize in 8 M urea the conformation of the microbial enzyme. A study was carried out to see if the guanidinium complex of either trypsin could demonstrate a changed specificity toward N-acyl-aminoacyl-p-nitroanilides. The results were entirely negative.

The requirement for calcium ion to stabilize two serine endopeptidases purified from pronase, which are homologous with mammalian chymotrypsin, has been recently described (2). The present report reveals the results of a similar study extended to the component in pronase which is closely homologous to bovine trypsin (3). As demonstrated below, this latter enzyme is more heat-labile and less stable in urea than its two genetically related companion proteins.

We described earlier the technique of purification of two stable pronase endopeptidases by incubation in 6 M guanidine HCl, wherein all other protein components were hydrolyzed to low molecular weight products (4, 5). Our attention was directed to the possibility that lower concentrations of this denaturant might permit the facilitated purification of other pronase enzymes. The unexpected observation of stability of the trypsin component in guanidine concentrations at 4 M or less as compared to the lability in 8 M urea led to the consideration that guanidine might be affecting this protein in an anomalous fashion. The possibility was examined that guanidinium ion could stabilize this protease under certain conditions. This proved to be the case as the following studies disclose.
Streptomyces griseus trypsin was purified as before (1, 6) from commercial pronase (Lots 900055, 000130, and 101185) obtained from Calbiochem. S. griseus guanidine-oc-tetradecanoylphorbol acetate was prepared as previously described (4). Bovine trypsin (Lot TH 11G) was obtained from Worthington Biochemical Corp. as a two step crystallized, salt-free, freeze-dried preparation. N\textsuperscript{\textcircled{\textdagger}}-Benzoyl-L-arginine ethyl ester, N\textsuperscript{\textcircled{\textdagger}}-benzoyl-L-arginine ethyl ester, N\textsuperscript{\textcircled{\textdagger}}-butoxyxycarbonyl-L-aspartic acid-benzyl ester, and N\textsuperscript{\textcircled{\textdagger}}-butoxyxycarbonyl-O-benzyl-L-serine were obtained from Schwarz-Mann. Eastman Organic Chemicals was the source for urea, reagent grade, was obtained from Mallinckrodt Chemical Works. It was further purified by dissolving in a minimal volume of boiling ethanol and thereafter allowed to crystallize at room temperature. After filtration, the crystals were dried under vacuum in a dessicator. Guanidine HCl was prepared as before (5) or obtained in the ultrapure form from Schwarz-Mann. The activities against Bz-Arg-0Et, N\textsuperscript{\textcircled{\textdagger}}-Benzoyl-L-arginine-\textbeta-nitroanilide were determined by the standard assay with the fluorogenic agent with bovine serum albumin as a standard (10). Protein concentrations of the bovine enzyme were determined by absorbance at 280 nm where \( \varepsilon_{280} = 14.4 \) (11). N\textsuperscript{\textcircled{\textdagger}}-Acetyl-L-aspartate-\textalpha-\textbeta-nitroanilide and N\textsuperscript{\textcircled{\textdagger}}-acetyl-L-serine-\textbeta-nitroanilide were prepared in the following manner. One gram of either N\textsuperscript{\textcircled{\textdagger}}-butoxyxycarbonyl-L-aspartic acid-benzyl ester or N\textsuperscript{\textcircled{\textdagger}}-butoxyxycarbonyl-O-benzyl-L-serine was dissolved in 15 ml of methylene chloride and added to a 5-ml methylene chloride solution containing 0.04 and 0.70 g of dicyclohexylcarbodiimide, respectively. Each solution was added 0.43 and 0.46 g of p-nitroaniline, respectively. After standing 24 hours at room temperature, the mixtures were filtered and the precipitates discarded. Each reaction mixture was concentrated in vacuo and washed with diethyl ether. The crude products were separately dissolved in a minimal volume of a solution of methylene chloride-trifluoroacetic acid (80:20, v/v) and treated with anhydrous hydrogen bromide for 90 min. Removal of the solvent left oily residues which were precipitated by the addition of ether. After washing with ether, the crude products were dissolved separately in 7-ml aliquots of dimethyl formamide containing 94 and 0.40 ml of triethylamine, respectively, to each of which 2 mg of enzyme was added. Each reaction mixture was allowed to stand at 24\textdegree C, and at intervals, 20-\mu l aliquots were removed for assay against Bz-Arg-0Et and Bz-Arg-\textbeta-NHNp. All activities were plotted as per- centages of initial activities.

The heat stability of pronase trypsin was studied in the presence and absence of urea as described in the Experimental Procedure. The following procedure was carried out to determine the specific cation which stabilized the enzyme against heat denaturation. Five milligrams of enzyme were dissolved in 1.0 ml of 100 mM glycine (pH 3.4) containing 10 m\textsuperscript{\textdagger}l EDTA. The solution was passed through a Sephadex G-25 column equilibrated with the above glycine solution. The fractions containing the eluted protein were collected. Aliquots (100 \mu l) containing about 0.4 mg of protein were added to separate tubes containing 30 \mu l of dif- ferent chloride salts at 0.1 M concentration. Immediately thereafter, 0.1-ml aliquots of enzyme solution were diluted in 0.9 ml of 10 mM Tris containing either 110 mM CaCl\textsubscript{2}; 11 mM EDTA; 1.1 mM urea; 11 mM CaCl\textsubscript{2}; 11 mM urea; 110 mM CaCl\textsubscript{2}; 8.8 M urea; 550 mM CaCl\textsubscript{2} 8.8 M urea; or 11 mM EDTA 8.8 M urea. The pH value of each diluent was 8.0. The solutions were allowed to stand at 24\textdegree C, and at intervals, 20-\mu l aliquots were removed for assay against Bz-Arg-0Et. All activities were plotted as percentages of initial activities.

The stability of pronase trypsin was studied in the presence and absence of 8 M urea. Fig. 1 demonstrates the results. The stability of the enzyme in Tris buffer at pH 8 is not affected by EDTA. The striking finding of these studies is the observation of the combined effects of EDTA and denaturant upon the protein; in this case, the rate of autolysis is such that 55% of the activity is lost within 2 min.

The results of these studies with the two smaller enzymes (2), the effect of EDTA on the protein was studied as a function of temperature. Fig. 2 reveals the results of this investigation. No effect of EDTA is seen at 24\textdegree C. However, at higher temperatures, the clear destabilizing effect of EDTA is demonstrable. Apparent metal-free enzyme was prepared according to the procedure outlined above and an analysis was carried out on the protective effect of various cations on the stabilities at 37\textdegree C and 49\textdegree C. Table 1 demonstrates that calcium was the specific metal ion required for this protein. Following these results, studies
Calcium levels diminish the rate of activity loss, and at a concentration of 0.5 mM CaCl₂, or with 10 mM EDTA. Stability of microbial trypsin in the presence (O-O) and absence (●-●) of EDTA. See text for details. All incubations were carried out at 23°C.

Table I

Stability of microbial trypsin in presence of various cations

| Cation | Preincubation at 24°C | Preincubation at 37°C | Preincubation at 4°C |
|--------|----------------------|----------------------|----------------------|
| None   | 100                  | 88                   | 0                    |
| Ca²⁺   | 100                  | 100                  | 100                  |
| Ba²⁺   | 81                   | 100                  | 0                    |
| Cd²⁺   | 74                   | 0                    | 0                    |
| Co²⁺   | 87                   | 0                    | 0                    |
| Cu²⁺   | 91                   | 0                    | 0                    |
| K⁺     | 87                   | 0                    | 0                    |
| Mg²⁺   | 71                   | 0                    | 0                    |
| Mn²⁺   | 98                   | 0                    | 0                    |
| Na⁺    | 92                   | 0                    | 0                    |
| Ni²⁺   | 98                   | 0                    | 0                    |
| Sr²⁺   | 98                   | 0                    | 0                    |
| Zn²⁺   | 87                   | 0                    | 0                    |

were carried out to see if calcium ion could prevent denaturation of the enzyme in 8 M urea. As depicted in Fig. 1, increasing calcium levels diminish the rate of activity loss, and at a concentration of 0.5 mM, this ion protects completely.

As described above, incubation of crude pronase in guanidine HCl at concentrations of 4 M or less did not result in loss of activity of the trypsin component. This implied that the trypsin remained in the native state in these solutions since two companion pronase serine endopeptidases are known to be active and stable in these concentrations of denaturant (4, 5), and would hydrolyze any unfolded species. Although clearly a rigorous generalization cannot be made, studies of unfolding of several well known proteins lead to the observation that 4 M guanidine HCl is at least equivalent in denaturant effect to the higher (8 M) urea concentration (12-18). This led to our consideration that there might be an anomalous effect of guanidine upon this protein, and we examined the possibility that this ion might bind to the enzyme at the site which interacts with arginyl guanidino groups of substrate molecules. The effect of varying guanidine HCl concentrations on the hydrolysis of Bz-Arg-NHNp was investigated. Table II demonstrates the results found with a 3 M concentration of the salt. After addition of denaturant, almost complete cessation of this activity is seen. Lower concentrations of guanidine yield lesser degrees of inhibition. Similar findings are observed with bovine trypsin but at a much lower guanidine HCl concentration. In contrast, no effect is noted on the activity of each enzyme against Ac-ONp. This selectivity of guanidine inhibition is not surprising; crystallographic studies of the bovine enzyme have demonstrated the putative binding site to be sufficiently distant from the reactive serine residue (19) so that an associated guanidinium molecule should not hinder hydrolysis of Ac-ONp. Plots of kinetic data revealed the inhibition of hydrolysis of Bz-Arg-NHNp by guanidine HCl in each enzyme to be competitive in nature. Table III lists the kinetic constants. As noted, the Michaelis constant of the bovine enzyme is about 20-fold greater than that of the microbial enzyme. Microbial trypsin demonstrates about a 3-fold greater affinity for guanidinium ion than does bovine trypsin.

Further studies were carried out to determine whether guanidine could actually stabilize each trypsin against denaturation in urea. Fig. 3 depicts the results. Guanidine (0.2 M) only diminishes slightly the rate of autolysis of the bovine enzyme in 8 M urea, whereas the microbial enzyme is completely protected. Calcium ion at a low concentration is required for this guanidine effect on microbial trypsin. With the latter enzyme, two possibilities can be entertained: either the effect of guanidine rests only...
TABLE III

Kinetic constants for trypsins

| Trypsin          | Bz-L-Arg-NHNp | Guanidine HCl (K_i) |
|------------------|---------------|---------------------|
|                  | \( K_m \) | \( V_{\max} \) | \( K_i \) |
| S. griseus       | 0.022 | 5         | 0.50 |
| Bovine           | 0.40  | 1         | 1.8  |

All assays were carried out in 10 mM Tris-HCl-5 mM CaCl_2 (pH 8.0) at 23°C. The concentration ranges of enzymes, N'-benzoyl-L-arginine-p-nitroanilide, and guanidine HCl were 0.8 to 20 mM, 5.6 to 450 \( \mu \)M, and 3.75 to 15 mM, respectively.

![Fig. 3. The stability of trypsins in 8 M urea. Left, Streptomyces griseus trypsin, 50 \( \mu \)g per ml; right, bovine trypsin, 450 \( \mu \)g per ml. All solutions contain 8 M urea-50 mM Tris-HCl (pH 8) with the following additions: 10 mM CaCl_2 ( ), 10 mM CaCl_2, 0.2 M guanidine-HCl (- - -); 10 mM CaCl_2, 500 \( \mu \)g per ml guanidine-stable chymoelastase ( ); 10 mM CaCl_2, 500 \( \mu \)g per ml guanidine-stable chymoelastase, 0.2 M guanidine-HCl ( - - - ); 10 mM CaCl_2, 500 \( \mu \)g per ml guanidine-stable chymoelastase, 0.5 M guanidine HCl ( - - - ); 10 mM CaCl_2, 500 \( \mu \)g per ml guanidine-stable chymoelastase, 1 M guanidine HCl ( - - - ) and 10 mM EDTA, 0.2 M guanidine HCl ( - - - ). All incubations were carried out at 23°C. Twenty-microliter aliquots were removed for the standard assays against 2-ml solutions of Bz-L-Arg-NHNp. See text for further details.](http://www.jbc.org/content/full/256/19/6143/F3)

![Fig. 4. Circular dichroism spectra of Streptomyces griseus trypsin. Spectrum of enzyme without denaturant or in 8 M urea and 450 mM CaCl_2 (---); spectrum of enzyme in 8 M urea (---); spectrum of enzyme in 8 M urea with 9 mM CaCl_2 and 0.2 M guanidine HCl (---). Each spectrum remained unchanged for 1 hour. See text for further details.](http://www.jbc.org/content/full/256/19/6143/F4)

on its inhibition of enzyme activity, or guanidine not only inhibits but also stabilizes the enzyme by shifting the conformational equilibrium toward the tightly folded native state. Incubation of microbial trypsin in 8 M urea-0.2 M guanidine HCl was carried out in the presence of a 12-fold molar excess of guanidine-stable chymoelastase purified from pronase (2). This latter protease is active and stable in the denaturant mixtures that were prepared for the following studies. As shown in Fig. 3, 0.2 M guanidine HCl incompletely protects against possible proteolysis of the trypsin by the chymoelastase in 8 M urea; at a higher (1.0 M) guanidine concentration, 90% of the initial activity is conserved after 2 hours. Excess guanidine-stable chymoelastase greatly accelerates the irreversible denaturation of microbial trypsin in 8 M urea in the absence of guanidine (Fig. 3); this excludes the remote possibility that microbial trypsin is unusually resistant to the action of the chymoelastase.

Circular dichroism studies of S. griseus trypsin revealed, in the absence of denaturant, conformational features indicative of a low amount of helix (Fig. 4). These conformational features are largely lost immediately after transfer of enzyme to 8 M urea. If 0.45 M calcium ion is also present with the urea, no change in the spectrum is noted. This confirms the stabilizing effect of calcium. When the spectrum was studied at a low calcium concentration (9 mM) with 0.2 M guanidine HCl, an unchanging intermediate conformation was noted. We believe this represents a stable equilibrium between denatured protein and fully inhibited native protein. In contrast to the studies with microbial enzyme, bovine trypsin was largely unprotected in 8 M urea by either 0.45 M CaCl_2 or by a 9 mM CaCl_2-0.2 M guanidine HCl mixture. We attempted to carry out similar spectral studies at pH 4.5, where, in the absence of denaturant, the microbial enzyme is stable and inactive. Neither 0.45 M CaCl_2 nor the 9 mM CaCl_2-0.2 M guanidine HCl mixture protected against unfolding in 8 M urea. This latter finding may be explained by the knowledge that bovine trypsin undergoes a conformational transition between pH values of 7 and 5 (20). In view of the close homology, such a transition may occur also with microbial trypsin and may result in poor binding of calcium and guanidine at an acidic pH. On the other hand, the results noted at pH 4.5 may represent only the inhibition of binding of cations due to the protonation of the liganding groups.

The binding of guanidine near the catalytic site suggested the possibility that the specificity of trypsin might be redirected. Liganded guanidinium ion could form a hydrogen bond either with the \( \beta \)-carboxyl group of a substrate aspartyl residue or with the hydroxyl oxygen of a substrate seryl residue. To this end, Ac-Asp-NHNp and Ac-Ser-NHNp were synthesized as model substrates. Neither microbial nor bovine trypsin was able to catalyze hydrolysis of either compound in the absence or presence of 0.2 M guanidine HCl.

**DISCUSSION**

The finding that calcium stabilizes this trypsin is not unexpected in view of the earlier description of calcium stabilization of the two smaller homologous components in pronase (2) and also of bovine trypsin (21-23). This enzyme is less stable in
denaturant than its two companion homologous endopeptidases (2); the present study demonstrates that the differences in stabilities in denaturant of these three proteins may be in part attributable to differences in affinity for calcium ion. The stabilization of this trypsin in 8 M urea by 0.5 M calcium ion makes it a potentially useful agent for protein structural studies. Recently the possible residues which have been associated with calcium binding in bovine trypsin have been tentatively identified (19). The sequences of S. griseus trypsin in regions of primary structure homologous to these bovine residues have yet to be reported.

This study demonstrates that the microbial enzyme is more stable in denaturant than its bovine homolog. This is not unexpected in view of the marked stability of companion proteases found in pronase (4, 5). Of great interest is the finding that low concentrations of guanidine counteract in part the denaturant effect of high concentrations of urea. This is explained by the present data to be due to guanidine binding at the specificity site of the microbial trypsin. Unequivocal proof of this interpretation would require specific crystallographic studies. Many investigators have demonstrated the inhibitory effect of alkyl guanidine derivatives on the action of bovine trypsin (24–28). The emphasis in the past has been placed on the cooperative manner of calcium ion stabilizing the S. griseus protein. The stabilizing effect of calcium is dependent upon the presence of calcium ion; if excess EDTA is also present, guanidine does not protect the microbial enzyme against urea denaturation (Fig. 3). Since guanidine in contrast to calcium stabilizes the enzyme in a manner independent of calcium, the present data demonstrate that an alkyl group is not required for the stabilizing effect of high concentrations of urea. This is explained by the marked stability of companion proteases for guanidine to prevent autolysis of bovine trypsin. It would ordinarily be expected, despite the observed rapid unfolding of this enzyme in urea, that any species in the native folded state would be strongly inhibited by this concentration of guanidine. However, the local site which binds guanidine may be so susceptible to urea effects that the affinity for guanidine is greatly reduced in urea even for the small fraction of molecules which are in the active native state. We have carefully examined the primary structures of the two trypsins to see if some further insight into this problem can be derived from the observed differences in sequences. One remote possibility has occurred to us and is offered in the following discussion. Smith and Shaw (29), in their studies with bovine trypsin, noted that an active product of autolysis, designated pseudotrypsin, was generated by cleavage between Lys-188 and Asp-189. The latter residue is the one which interacts specifically with the positively charged groups of substrate residues (19). Pseudotrypsin was demonstrated to have marked diminution in activity and marked increase in kcat toward Bz-Arg-OEt, and also a great decrease in affinity toward the specific inhibitor, benzamidine, when compared to the precursor enzyme, α-trypsin. Little change in activity toward a poor nonspecific substrate, Nα-acetyl-L-tyrosine ethyl ester was noted. These results suggest that pseudotrypsin would have a very low affinity for guanidine. The present kinetic studies with model substrates should not detect any significant contribution of activity from possible contaminating amounts of bovine pseudotrypsin. To our knowledge, no description of possible protease activity in pseudotrypsin has been reported. If protease activity is present in that trypsin species, it could readily explain the present results with bovine trypsin. Since S. griseus trypsin has a valuable residue in place of Lys-188 (3), there cannot be an entity such as microbial pseudotrypsin, and such a species with a low affinity for guanidine is not present.

The failure to demonstrate activity against Ac-Aap-NH2 or Ac-Ser-NH2 emphasizes how exacting the requirements are for the binding and hydrolysis of specific substrates by trypsin.

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