Self Purification of Two Serine Endopeptidases*

(Pronase/6 M guanidinium chloride/chymotrypsin/subtilisin)

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ABSTRACT We have reported that a serine protease from Pronase, homologous with bovine chymotrypsin, is both active and stable in 6 M guanidinium chloride. The present investigation examined the possibility that this unique property might be used to permit the enzyme to engage in its own purification by cleaving companion proteins to low-molecular-weight products. Analysis with model substrates of the several specific activities that were originally present revealed that only the activity against *N* α -acetyl-L-tyrosine ethyl ester was demonstrable after incubation for 100 hr in the denaturant. After a moderate loss within the first 24 hr, the remaining activity against this ester was conserved for many days thereafter. Pronase was routinely incubated for 1 week at 22° in 6 M guanidinium chloride at pH 8.0 where the esterases showed maximal activity. Analysis of the products of incubation revealed unexpectedly the presence of two serine proteases that were easily separated. After purification to homogeneity these components proved themselves to be the previously demonstrated subtilisin-like and stable chymotrypsin-like enzymes. The only amino-terminal residue of the chymotrypsin-like enzyme is isoleucine, as it is in the earlier, conventionally purified product. The migration of the single band of this enzyme during acrylamide gel electrophoresis was the same whether purified by the past or present technique. No free amino-terminal group was demonstrable in the subtilisin-like enzyme. This study presents a unique and rapid technique for isolation of these proteases, with the first reported purification to homogeneity of the subtilisin-like component. These enzymes may be useful as probes for local relaxations of conformation in substrate proteins. Furthermore, they may contribute to the preparation of enzyme-free non-protein macromolecules.

We recently described the complete purification of three serine endopeptidases found in Pronase that are homologous with chymotrypsin (1). Also described was the partial purification of a serine protease homologous with subtilisin. A second communication demonstrated that one of the chymotrypsin-like enzymes was uniquely both stable and active in 6 M guanidinium chloride (Gnd·HCl) (2). This enzyme hydrolyzed *N* α -acetyl-L-tyrosine ethyl ester (AcTyrOEt) in this denaturant and also hydrolyzed casein at a faster rate in the presence than in the absence of 6 M Gnd·HCl. Ovalbumin was hydrolyzed extensively only in the denaturant. These results suggested that selective unfolding of substrate

proteins enhanced the rate and extent of proteolysis by the very stable endopeptidase. Circular dichroism studies revealed no differences in the spectrum of the enzyme in the presence or absence of 6 M Gnd·HCl or 8 M urea (2). The very natural consideration arose, therefore, that the marked stability and catalytic properties of this enzyme might be used for its facilitated purification. For, if Pronase were incubated in denaturant, the possible selective hydrolysis of the other protein components could leave only the stable protease in the native macromolecular state. As will be demonstrated below the principle of the technique was experimentally confirmed; however, not one, but two enzymes were found to be stable in 6 M Gnd·HCl.

MATERIALS AND METHODS

Pronase (grade B) was obtained from Calbiochem; two lots (numbers 000333 and 101185) were used for these studies. AcTyrOEt and *N* α -benzoyl-L-arginine ethyl ester (BzArgOEt) were obtained from Mann Research Labs. Gnd·HCl was prepared as described (2) or obtained in the ultrapure form from Schwarz-Mann; no differences were noted in the results described below when either preparation was used. Casein was obtained from Difco Corp. and purified (1). Pre-swollen microgranular carboxymethyl (CM)-cellulose (Whatman CM-52) was obtained from Reeve Angel Co. Sephadex G-25 and G-75 were obtained from Pharmacia Fine Chemicals. Polyamide Layer, a product of the Cheng Chin Trading Co., Ltd., was purchased from Gallard-Schlesinger. The 15 × 15 cm sheets were quartered before use for analysis of dimethylaminonaphthylsulfonyl (dansyl) amino-acid residues. [³²P]Di-isopropylphosphorofluoridate (80 Ci/mol) was obtained from Amersham-Searle. All other chemicals were of reagent grade.

Preparation of Enzymes. 2 g of Pronase was dissolved in 10 ml of 10 mM Tris·HCl (pH 8.0)–10 mM CaCl₂–7.5 M Gnd·HCl at 0°. The scant amount of insoluble material was removed by centrifugation at 0°. To the dark-brown supernatant, 10 mM Tris·HCl (pH 8.0) containing 10 mM CaCl₂ was added to a final total volume of 12.5 ml. Thereafter, the contained solution was wrapped in aluminum foil (to avoid the effects of light) and allowed to stand at room temperature (22°) for 1 week. During this time the pH of the solution dropped to between 7.2 and 7.4. Thereafter, the scant amount of precipitate formed during the incubation was removed by centrifugation at 0° and the supernatant material was passed in 2.5-ml aliquots through a Sephadex G-25 column (1.5 × 25 cm) equilibrated with 5 mM Tris·HCl (pH 7.6) containing 5 mM CaCl₂. An initial peak with excluded

Abbreviations: Gnd·HCl, guanidinium chloride; AcTyrOEt, *N* α -acetyl-L-tyrosine ethyl ester; BzArgOEt, *N* α -benzoyl-L-arginine ethyl ester; dansyl, dimethylaminonaphthylsulfonyl.

* This is Paper no. III in the series, "The Proteolytic Enzymes of the K-1 Strain of *Streptomyces griseus* obtained from a Commercial Preparation (Pronase)"; Papers I and II in this series are refs. 1 and 2.

FIG. 1. Stability of Pronase components in 6 M Gnd·HCl. Activity against AcTyrOEt (○—○); activity against BzArgOEt (●—●). 5- μ l Aliquots were removed for the rate assays; the only denaturant in each assay was the amount transferred in the enzyme aliquot. The total volume in each assay was 3.0 ml.

material was noted, followed by a broad profile of absorbancy at 280 nm. The earliest fractions, which contained all of the activity against AcTyrOEt, were combined and concentrated by Diaflo ultrafiltration (Amicon Corp.) through a UM-2 membrane. The diffusate, which was clear and colorless, gave a very low absorbance at 280 nm. The retentate was dark brown and clear. A scant, brown, amorphous, and gelatinous precipitate could be seen on the membrane surface. It was easily washed off with distilled water and discarded.

Chromatography. The dark-brown retentate from the ultrafiltration was processed in two ways. In most runs it was passed through a CM-cellulose column (1.1 $\times$ 7 cm) equilibrated with 5 mM Tris·HCl (pH 8.0) containing 5 mM CaCl₂. After a period of flow with this buffer, elution was continued with a linear gradient from 150 ml of the buffer only and 150 ml of the buffer containing 0.25 M NaCl. In a few runs the retentate was passed directly through a Sephadex G-75 column (1.1 $\times$ 195 cm) equilibrated with 10 mM Tris·HCl (pH 8.0) containing 10 mM CaCl₂. Chromatographic components obtained from the CM-cellulose column were concentrated by ultrafiltration as above and passed through the above Sephadex G-75 column for further analysis. Effluent fractions were monitored by the measurement of absorbance at 280 nm, except in occasional runs where a large amount of protein had been applied. In these latter studies, the absorbance at 295 nm was followed. As will be demonstrated below, during the chromatography of the Pronase enzymes, a low molecular weight substance, which was brown in color, was also noted. The migration of this material was followed by the absorbance at 320 nm. All chromatography was done at 4°. The fractions containing each enzyme at the final step of purification were colorless.

Measurement of Catalytic Activity. The extent of casein digestion and the activities against AcTyrOEt and BzArgOEt were measured by techniques described earlier (1, 3). Though in different experiments different aliquot volumes of enzyme solutions were used, all data for esterase activities of chromatographic fractions have been normalized to rates for 50- μ l aliquots.

Gel Electrophoresis. Electrophoresis in polyacrylamide gel was performed in a Canaco model 12 apparatus. The amount of protein applied was determined according to the method of Lowry *et al.* (4) with bovine serum albumin as standard.

All studies were done at pH 4.3; the buffer composition was that designated by the *Disc Electrophoresis* bulletin of the Canal Industrial Corp., New York. Because of diffusion of the chymotrypsin-like enzyme during the destaining period (1, 2), the gels containing this enzyme at the end of each run were treated by the technique of Lenard, where trichloroacetic acid was used to precipitate and denature the protein (5). The gel was destained with 10% acetic acid. The subtilisin-like enzyme was denatured and subsequently stained with Coomassie blue by the earlier technique (1).

Identification of Amino-Terminal Residues. The amino-terminal residues of the purified proteins were identified by reaction with dansyl chloride (6). The technique was modified by substituting acetone for the acetonitrile. The results were analyzed by two-dimensional chromatography (7) on the quartered polyamide sheets.

RESULTS

The esterase activities against AcTyrOEt and BzArgOEt were followed in crude Pronase incubated in 6 M Gnd·HCl. As seen in Fig. 1 the activity of Pronase trypsin (1, 3, 8, 9) is lost fairly rapidly, so that by 4 hr it is no longer discernible. In contrast the activity against AcTyrOEt drops to about 75% of the initial activity but thereafter remains unchanged. This activity remained constant for over a week under the conditions of incubation. The level of residual activity was unexpected; the contribution to the activity against AcTyrOEt by the previously demonstrated, stable chymotrypsin-like enzyme (1, 2) could not have exceeded 30% of the total. These results strongly suggested either some kind of activation of the stable enzyme, or the conservation of one of the other enzymes with activity against the neutral ester. After the incubation the crude Pronase solution was tested for activity against a series of other model substrates. These included L-leucine-*p*-nitroanilide, glycyl-L-phenylalanine, hippuryl-L-lysine, and carbobenzyloxy-glycyl-L-leucine. The results were entirely negative.

Passage through a CM-cellulose column of the auto-lyzed Pronase solution as described above yielded two large peaks with activity against AcTyrOEt (Fig. 2). The first peak is completely excluded from the column, whereas the second peak is only eluted after the start of a sodium chloride gradient. These results do not reflect saturation of binding

FIG. 2. Chromatography through CM-cellulose of guanidine-stable components from Pronase. Fractions of 4.2 ml were collected. See text for details.

FIG. 3. Gel filtration through Sephadex G-75 of guanidine-stable components from Pronase. *Panel A*: filtration pattern of material excluded from a Sephadex G-25 column after incubation for 1 week in 6 M Gnd·HCl; protein elution profile determined by absorbance at 295 nm (●—●). Fractions of 2.5 ml were collected. *Panel B*: filtration pattern of material in fractions excluded from CM-cellulose column (see Fig. 2). Protein elution profile was determined by absorbance at 280 nm (●—●). Fractions of 3.2 ml were collected. *Panel C*: filtration pattern of material from fractions with activity against AcTyrOEt in the run through Sephadex G-75 illustrated in *Panel B*. Protein elution profile was determined by absorbance at 280 nm (●—●). Fractions of 3.0 ml were collected.

sites on the column with excess applied protein for, if the protein in the excluded fractions was reapplied to the column, all of the activity against AcTyrOEt was completely excluded. Thus, as suspected from the above studies, two different esterases were conserved when incubated in the denaturant. Most of the dark-brown material was excluded from the column, though a long trail of absorbance at 320 nm can be seen.

Passage of whole autolyzed Pronase through a Sephadex G-75 column again demonstrated two peaks with activity against AcTyrOEt (Fig. 3A). The pattern of this activity is very similar to that seen in the filtration pattern of whole native Pronase (see Fig. 2 of ref. 1). Closely following and overlapping the fractions with esterase activity was a symmetrical peak that was brown in color, as noted by the absorbance at 320 nm. Almost completely excluded from the column was an initial small peak also with absorbance at 320 nm. The proteolytic activity against casein is confined to, and spans, the fractions with esterase activity.

The protein in the excluded fractions of the CM-cellulose run was concentrated by ultrafiltration and passed through a Sephadex G-75 column. The filtration pattern (Fig. 3B) is very similar to that seen with whole autolyzed Pronase except that the second peak with activity against AcTyrOEt has been completely removed. It appeared that this second peak was probably identical to the peak retained by the CM-cellulose column. This was confirmed by demonstrating that the protein associated with this second peak filtered through the Sephadex column at a volume associated with the missing peak. It is to be noted that the proteolytic activity against casein in Fig. 3B is confined to the fractions associated with the single peak of activity against AcTyrOEt.

The protein associated with the filtration fractions with esterase activity in Fig. 3B was concentrated and passed again through the Sephadex G-75 column. The symmetrical pattern of a single peak—as measured by absorbance at 280 nm, esterase activity against AcTyrOEt, and proteolytic activity against casein—is seen (Fig. 3C). This material was saved and stored at 0° for further studies.

An analysis was made of the sequences around the reactive serine residue of each enzyme by previously described tech-

niques after reaction with [³²P]di-isopropylphosphorofluoridate (1). The protein associated with the peak in Fig. 3C gave a pattern of [³²P]di-isopropylphosphoryl peptides like that of subtilisin, whereas the protein retained by the CM-cellulose column gave a pattern of radioactive peptides like that of chymotrypsin.

Fig. 4 demonstrates the acrylamide gel patterns of the purified proteins. Each enzyme appears to be homogeneous. The subtilisin enzyme was studied as the di-isopropylphosphoryl derivative because the native enzyme was unstable at the low pH of the gel run. The migration of the chymotrypsin-like enzyme purified by the present technique is identical to that of the enzyme purified by the past conventional method (1).

FIG. 4. Acrylamide gel electrophoresis of purified guanidine-stable components of Pronase. The origin of each run was at the bottom. Electrophoresis was done at room temperature at 3 mA per tube for 3 hr. *Tube 1*: stable chymotrypsin-like enzyme purified by previously described techniques (1); 10 μ l containing 50 μ g of protein was applied. *Tube 2*: stable chymotrypsin-like enzyme purified by the present technique (material obtained from fractions containing the activity against AcTyrOEt with the NaCl gradient during chromatography through CM-cellulose as seen in Fig. 2); 10 μ l containing 60 μ g of protein was applied. *Tube 3*: 10 μ l each of the solutions analyzed in tubes 1 and 2 were mixed and the total volume applied. *Tube 4*: 20 μ l containing 55 μ g of the di-isopropylphosphoryl derivative of the subtilisin-like enzyme was applied (prepared from fractions containing the esterase activity in Fig. 3C).

FIG. 5. Dansyl amino-acid patterns obtained from the acid hydrolysates of the stable proteases that were treated with dansyl chloride. Photographs of fluorescence during exposure to an ultraviolet lamp (H38-4JM mercury flood lamp with Kopp filter no. 41 UV, Black Light Eastern Corp., New York, N.Y.). 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 glacial acetic solution from bottom to top (7). *Left panel*: the pattern obtained from the stable chymotrypsin-like enzyme purified by conventional means (1). *Middle panel*: the pattern obtained from the stable chymotrypsin-like enzyme purified by the technique described in this report. *Right panel*: the pattern obtained from the subtilisin-like enzyme contained in fractions of the filtration peak demonstrated in Fig. 3C. Material derived from 2 nmol of protein was applied to the left and middle panels and from 5 nmol of protein to the right panel. The ϵ -amino-dansyl-lysine arcs in the left and middle panels are partially merged with the dimethylaminonaphthylsulfonic acid streak.

Fig. 5 demonstrates the results of the analysis of the amino-terminal residues. The chymotrypsin-like enzyme whether purified by the past or present technique reveals only the pattern of dansyl-isoleucine. The identity of this amino acid was confirmed by analysis with several dansyl standards. Furthermore, a mixture of hydrolyzed dansyl proteins isolated by the two techniques demonstrated excellent superposition of patterns. The subtilisin-like enzyme reveals the pattern of ϵ -amino-dansyl-lysine, but not of any amino-terminal residue. These findings strongly support the other evidence for homogeneity of the purified enzymes.

DISCUSSION

The Pronase proteases investigated, besides being of interest because of their stability, have the potential of broad application. They may be useful for the analysis of other polypeptides, not only in the determination of primary structure but also as probes for the sequential regional unfolding of proteins exposed to increasing concentrations of denaturant. Furthermore, the present principle of purification in Gnd·HCl may be applied with these enzymes to other protein mixtures where there is noted a significantly selective difference of stability in denaturant. These proteases may also be useful in the preparation of such macromolecules as polynucleotides or polysaccharides so that these nonprotein polymers would be free of contaminating protein. The low cost of Pronase and the facility of the present scheme of purification make the two serine enzymes readily available.

The stability of the two proteases is extraordinary. They cannot be like earlier examples of complete unfolding of proteins in denaturant with reversible renaturation upon dilution, for it has already been demonstrated that the chymotrypsin-like enzyme is active in the denaturant and will hydrolyze randomly coiled molecules (2). Therefore, the subtilisin-like enzyme must also stay in or very close to the native state. It remains to be shown that this enzyme is also active in the denaturant.

It was pointed out earlier that the losses in the subtilisin-like enzyme during the conventional technique of purifica-

tion may have been due to the low pH (1). The subtilisins are known to be unstable in acid solutions, up to pH 4 (10). Pronase subtilisin may be even more labile, so that the prolonged exposure at pH 5 could have caused extensive autolysis. The maintenance of the pH between 7.2 and 8.0 in all steps of the present study may have been a significant feature in stabilizing the enzyme despite the fact that the enzyme is fully active in this higher pH range (1). Acid lability was noted in the present study during acrylamide gel electrophoresis, requiring the use of the di-isopropylphosphoryl derivative to demonstrate homogeneity.

It has been well documented that the subtilisins are stable in urea-containing solutions (10–12). A recent study of the stability of subtilisin Carlsberg demonstrated very slight changes in the reduced viscosity when the protein was transferred from denaturant-free solutions into 6 M Gnd·HCl (12). These studies were performed at pH 8. It was not clear whether with time there were further changes in the viscosity, or whether autolysis was noted.

The assumption that Pronase subtilisin is closely related to the other subtilisins remains to be validated. The homology is based entirely upon the similarity of apparent molecular weights and upon the probable common sequence, Thr-Ser-Met, around the reactive seryl residue (1). Whether the Pronase enzyme shows more extensive similarity with the defined subtilisins and is also free of disulfide bonds must be determined.

The present purification sequence has been performed many times. The yield of the chymotrypsin-like enzyme appears to be fairly constant, with a recovery between 90 and 100%. In contrast, the yield of the subtilisin-like enzyme varies in a range from an amount presently reported (Fig. 3) to about 50% of this amount. This represents an estimated recovery of 40–80%. The variance appears to be due to the first steps of purification, where the enzyme is briefly kept at 0° in 7.5 M Gnd·HCl, for the activity against AcTyrOEt obtained at 24 hr does not change during the next 3 days. In one instance when Pronase was left in 7 M Gnd·HCl at 22° for a week, a fairly good recovery of the chymotrypsin-like enzyme

was obtained, whereas the subtilisin enzyme was almost completely lost. In another instance, when the temperature was elevated to 30° for an indeterminate time, the yield of the subtilisin-like enzyme was again much lower. These preliminary observations suggest that the prescribed conditions of exposure to Gnd·HCl are close to tolerance for Pronase subtilisin, and that perhaps incubation with a lower Gnd·HCl concentration may routinely give better yields of this enzyme. It must be recalled that about 25% of the activity against AcTyrOEt must be lost by the present procedure since the smallest chymotrypsin-like enzyme is not stable in 6 M Gnd·HCl (2).

The chymotrypsin-like enzyme appears to be entirely protected in the present study, even to the apparent exclusion of limited autolysis. Admittedly, the possibility of an undetected loss of a carboxyl-terminal fragment has not been excluded. It must be emphasized again that only one of the Pronase enzymes homologous with bovine chymotrypsin demonstrates the unique stability in denaturant (2). Past studies with bovine chymotrypsin and subtilisin BPN' have revealed totally dissimilar primary and tertiary structures (13-16), except for a common "charge transfer system" at the active site (16, 17). The study of these two Pronase enzymes, because of their probable different genetic origins, may lead to the discovery of some common structural features that give each protein its marked stability. We have now commenced a collaborative effort in the determination of the primary structure of the stable chymotrypsin-like enzyme.

The nature of the low-molecular-weight brown material in Pronase is unknown. It has no peak of absorbancy at either 260 or 280 nm. This suggests that it is not a polynucleotide or a polypeptide with aromatic residues. Studies are now in progress to see if this molecule is important in the stabilization of the two proteases. It is not even known if the brown material is a microbial product or only a broth constituent for the commercial growth of *Streptomyces griseus*.

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