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Studies of the Heterogeneity of *Streptomyces griseus* Protease. Isolation and Characterization of an Alkaline Serine Protease from Commercial Pronase-P Derived from *Streptomyces griseus* K1

CARL-AXEL BAUER and BO LÖFQVIST

Biochemistry 1, Chemical Centre, P.O.B. 740, S-220 07 Lund 7, Sweden

1. A proteolytic enzyme has been mildly isolated from Pronase-P by gel filtration on Sephadex G-75 superfine. The yield was almost optimal and the specific activity of the enzyme was constant throughout the isolation procedure and storage.

2. Analytical polyacrylamide gel electrophoresis at pH 6.8 and 8.5 failed to reveal any impurities in the preparation. When the enzyme was concentrated 5 – 10 fold, two minor contaminents could be detected. They were estimated by staining to constitute less than 2 % and by enzymatic activity less than 1 %.

3. The binding of Ca^{2+} to the enzyme has been found to be non-stoikiometric, and leading to a higher electrophoretic mobility with increasing Ca^{2+}-content in the buffer. In Ca^{2+}-free media the enzyme has an isoelectric point just above 7.

4. Molecular weight was estimated to 18 000. The most striking feature of the amino acid analysis was that no lysine was detected.

5. The enzyme was shown to be most stable in Ca^{2+} containing buffers of neutral pH. In such buffers the enzyme was also stable in the pH-range 5 – 11, in this respect being similar to elastase. The pH-optimum of the enzyme towards glutarylphenylalanine *p*-nitroanilide was between pH 10 and 11.

6. The enzyme was inhibited completely by diisopropylfluorophosphate, and partly by inhibitors normally considered to be site-specific for either chymotrypsin or elastase.

7. The enzyme was highly active towards acetyl-L-tyrosine ethyl ester, carbobenzoxy-tyrosine *p*-nitrophenyl ester, and glutaryl-L-phenylalanine *p*-nitroanilide. It was also active towards acetyl-alanyl-alanyl-alanine methyl ester and congo-red elastin as well as towards many other substrates. The activity towards casein at pH 7.5 and pH 10 was approximately identical. Amidase activity of the enzyme was low.

8. The enzyme was classified as an alkaline serine protease, with broad substrate specificity showing similarities with both chymotrypsin and elastase. The results are discussed in relation to earlier investigations on this and similar enzymes.

The heterogeneity of Pronase, the extracellular proteolytic enzyme preparation from *Streptomyces griseus*, has been the subject of several investigations.[1–3] The most extensive fractionation of Pronase so far has been achieved by polyacrylamide gel electrophoresis.[3] By this method 14 enzymatically active protein components were resolved: 8 showing protease activity, 5 showing activity towards LNA,* while one component probably corresponded to the carboxypeptidase present in Pronase.[1] Only a few of the Pronase components, mainly some DFP-sensitive proteases, have been isolated and characterized further.[4–12] At present there is evidence for at least four DFP-sensitive proteases, three with molecular weights between 15 000 and 20 000,[4–12] and one with a molecular weight of approximately 28 000.[8,10] One of these enzymes, the "*Streptomyces griseus* trypsin", with a molecular weight of 20 000, is well characterized.[4–8] Recently, the other three DFP-sensitive enzymes were reported to show elastase-like activities.[10]

Although an acceptable fractionation of small amounts of Pronase components can be obtained by preparative polyacrylamide gel electrophoresis, it is not possible to isolate enough quantities for characterization purposes by this method.[3] In this laboratory, we have therefore developed a method for fractionating Pronase by gel filtration on Sephadex G-75 superfine, bead size $> 40\ \mu$.[13] Pronase is thereby separated into 7 enzymatically active peaks, designated A–G. Only two of these peaks, E and G, are homogeneous on polyacrylamide gel electrophoresis, and correspond to bands 4 and 3, respectively, of the earlier electrophoretic studies.[3] Since fraction E represents the comparatively well characterized "*Streptomyces griseus* trypsin",[3–8] we have chosen to study fraction G, one of the low molecular weight, DFP-sensitive enzymes. We have designated this enzyme SGP3, because of its migration in our high resolution electrophoretic separation system.[3]

Studies by Löfqvist and Sjöberg[3] showed that SGP3 is identical to the "PNPA hydrolase I" described by Wählby,[4,5,9] and to the "alkaline protease a" described by Narahashi.[1] Wählby showed that the amino acid sequence around the reactive seryl residue of "PNPA hydrolase I" was similar to that of chymotrypsin. Narahashi describes a purification of "alkaline protease a", but hardly gives any characterization data. Johnson and Smillie isolated an enzyme called "SGPA", and partly studied its amino acid sequence.[11,12] "SGPA" was found to be identical to ,,PNPA hydrolase I",[14] and should thus correspond to SGP3. Finally, Gertler and Trop have purified and partly characterized three "elastase-like enzymes from Pronase", of which "Enzyme II" corresponds to "PNPA hydrolase I",[10] and thus also to SGP3.

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* *Non-standard abbreviations.* Acetyl, Ac; *N*-acetyl-L-tyrosine ethyl ester, ATEE; *N*-benzoyl-L-arginine methyl ester, BAME; *N*-benzoyl-D,L-arginine-*p*-nitroanilide, BANA; carbobenzoxy, CBZ; diisopropylfluorophosphate, DFP; glutaryl-L-phenylalanine-*p*-nitroanilide, GPNA; methyl ester, ME; *p*-nitrophenyl ester, *p*-NPE; 4-phenyl-azo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine.$2H_2O$ is designated collagenase substrate A; tosyl ... chloromethyl ketones, T ... CK; L-Leu-β-naphthylamide, LNA.

Buffer I: 0.03 M borate buffer, pH 7.5, 0.03 M in $CaCl_2$ and containing 0.02 % NaN_3. Buffer II: buffers 0.3 M in H_3BO_3, 0.15 M in cacodylic acid and 0.1 M in acetic acid, adjusted to a given pH with NaOH. The enzyme studied throughout this investigation is called *Streptomyces griseus* protease 3, and is abbreviated SGP3.

Though SGP3 has been subject of some earlier studies, no extensive characterization of the enzyme has been performed by earlier investigators, and further some of their results are highly contradictory. This could partly be due to the fact that hereto used purification procedures have involved conditions, where the enzyme is unstable. Accordingly, figures concerning activity and yield, if presented, often show big losses.

The aim of this work has therefore been to mildly isolate the enzyme, with full control of specific activity and yield. The enzyme has, after this isolation, in many respects been more extensively characterized than earlier, and the results are discussed in relation to earlier investigations on this and similar enzymes.

EXPERIMENTAL

Materials

Enzyme. Pronase-P was obtained from the Kaken Chemical Company, Tokyo, (lot number 592045).

Inhibitors. ^{32}P-Diisopropylfluorophosphate was obtained from the Radiochemical Centre. Tosyl chloromethyl ketones of L-alanine, L-leucine, L-lysine, L-phenylalanine, and L-valine were purchased from Cyclo. 1-Bromo-4-(2,4 dinitrophenyl)-butan-2-one and acetylprolylalanylprolylalanine chloromethyl ketone * were kindly donated by Professor Elkan Blout, Harvard Medical School, US.

Enzyme substrates. (All low-molecular substrates were chromatographically homogeneous.)

High molecular weight substrates. Casein of Hammarsten grade was obtained from Merck AG, azocoll from Calbiochem AG, and congo-red elastin from Sigma Chemical Co.

Methyl and ethyl esters. *N*-Acetyl-L-tyrosine ethyl ester, *N*-benzoyl-L-alanine methyl ester, and *N*-benzoyl-L-arginine methyl ester.HCl were purchased from Sigma. *N*-Acetyl-L-alanine methyl ester, *N*-acetyl-L-alanyl-L-alanine methyl ester, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester, and *N*-benzoyl-glycine methyl ester were all purchased from Cyclo Chemical Co.

p-*Nitrophenyl esters.* *N*-Carbobenzoxy-L-alanine-*p*-nitrophenyl ester was obtained from Cyclo, *N*-carbobenzoxy-glycine-*p*-nitrophenyl ester, and *p*-nitrophenyl acetate from Sigma. *N*-Carbobenzoxy-nitrophenyl esters of L-leucine, L-proline, and L-tyrosine were products of Fluka AG.

Amides. *N*-Acetyl-L-alanine amide, *N*-carbobenzoxy-glycyl-L-leucine amide, and *N*-carbobenzoxy-glycyl-L-tyrosine amide were all from Cyclo. *N*-Carbobenzoxy-glycyl-L-phenylalanine amide was purchased from Sigma.

Other low-molecular weight substrates. *N*-Benzoyl-D,L-arginine-*p*-nitroanilide.HCl, *N*-carbobenzoxy-glycyl-L-leucine, and 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine.$2H_2O$ were obtained from Fluka AG. Glutaryl-L-phenylalanine-*p*-nitroanilide was purchased from Merck AG, glutaryl-L-phenylalanine-β-naphthylamide from Mann Research Laboratories, Inc., and L-leucine β-naphthylamide.HCl from Sigma.

Sephadex G-75 superfine (lot number 9070), and G-25 medium were obtained from Pharmacia Fine Chemicals AB. Bio-Rex 70 (200 – 400 mesh) was obtained from Bio-Rad Laboratories. All other chemicals used were of reagent grade or better.

Preparative methods

Gel filtration on Sephadex G-75. Pronase-P, 1.3 g, was dissolved in 16 ml of buffer I and was immediately applied to a Sephadex G-75 column (10 × 90 cm) and eluted at a flow rate of 60 – 70 ml/h according to Klevhag and Löfqvist.[13] A Uvicord (type 4701 A, LKB) set at 280 nm with LKB recorder and collector was used to monitor and collect the eluate. The protein fractions were stored at 4°C. Unless otherwise specified, these or concentrated solutions were used for characterization purposes.

* This preparation was not of the highest purity.

Ultrafiltration was used for concentration purposes and was carried out in an Amicon diaflo apparatus equipped with UM-05 filter.

Change of medium on Sephadex G-25. 7 ml concentrated enzyme solution (absorbance at 280 nm 1.0 – 2.0) was applied to a Sephadex G-25 column (2.5 × 40 cm), and eluted at 40 ml/h with 0.25 M NH_4HCO_3 in glass-distilled water, pH 7.7.

Lyophilization. The enzyme solution (in 0.25 M NH_4HCO_3) was frozen and repeatedly lyophilized to constant weight. Since the lyophilization procedures was found to occasionally cause heterogeneity, each batch was tested for homogeneity by electrophoresis on analytical polyacrylamide gels. Heterogeneous batches were discarded. The use of lyophilized enzyme for characterization was restricted to cases, where it could not be avoided.

Chromatography on Bio-Rex 70 was carried out essentially as described by Johnson and Smillie.[11] SGP3 (10 mg) or Pronase-P (200 mg) was dissolved in 3 ml of 0.05 M cacodylic-NaOH buffer, pH 6.0, and was applied to a Bio-Rex 70 column (2 × 50 cm). The column was developed with 12 l of cacodylic-NaOH buffer, pH 6.0, followed by 2 l of 0.05 M phosphate buffer, pH 7.5, and 2 l at pH 8.0, which finally released SGP3.

Assays of purity

Preparative polyacrylamide gel electrophoresis. Sample of 100 μl of SGP3 (0.10 – 0.25 mg) or Pronase-P (2 mg), active or ^{32}P-DFP-inhibited, in sucrose stabilized 0.38 M borate – 0.01 M Ca^{2+}-buffer, pH 6.8, were applied to preflushed gels and analyzed according to Löfqvist and Sjöberg.[3]

Analytical polyacrylamide gel electrophoresis as described by Löfqvist and Sjöberg[3] was performed on each SGP3 preparation in three different buffer systems. The buffers used were (a) 0.38 M borate – 0.01 M Ca^{2+} pH 6.8, (b) 0.02 M cacodylate – 0.015 M tris buffer, pH 6.8, and (c) 0.005 M tris – 0.04 M glycine buffer, pH 8.5. Samples either of 5 or 20 μl of SGP3 (0.1 – 40 μg) or 5 μl of Pronase-P (100 μg) were applied to the gels, and both anodic and cathodic migrations were performed.

Determination of ^{32}P-DFP incorporated into SGP3 in Pronase and in purified SGP3. The serine enzymes of Pronase were inhibited with ^{32}P-DFP as described under inhibition assays below. It was specially checked that the GPNA-activity of Pronase was zero. Pentaethylenhexamine was also added (final concentration 10^{-3} M) in order to inhibit the Zn^{2+}-dependent enzymes, which constitute the rest of the enzymes of Pronase (Löfqvist, B., unpublished). Precautions were thus taken to minimize the digestion. The inhibited Pronase components were separated on a preparative polyacrylamide gel as described above. After electrophoresis, the gel was cut in 1.5 mm thick discs, which were allowed to dry i vials, and 5 ml dioxan containing 0.8 % omnifluor (New England Nuclear) was added. The radioactivity of the ^{32}P-DFP-labelled components was determined in a Nuclear-Chicago Mark 1 liquid scintillation counter, and the number of SGP3 molecules in Pronase was calculated. No significant leakage of ^{32}P-DFP-inactivated SGP3 from the gels could be detected. The efficiency was approximately 82 %. Determination of ^{32}P-DFP incorporated into purified SGP3 was carried out as described above for SGP3 in Pronase but without pentaethylenhexamine.

Determination of protein. The extinction coefficient for the enzyme at 280 nm, E (1 %, 1 cm), was determined by measuring the absorbance of an enzyme solution in 0.25 M NH_4HCO_3. A known volume of the solution was then repeatedly lyophilized to constant weight (more than 98 % dry substance). The extinction coefficient was calculated by extrapolation to 1 % protein content.

Amino acid analyses. Amino acid analyses were carried out by the analytical division of the Institute of Biochemistry, Uppsala, Sweden, as described by Eaker.[15] Samples (both performic oxidized and unoxidized) were hydrolyzed for 24 h. Tryptophan was estimated by the method of Edelhoch.[16]

Optimal conditions and stability of the enzyme

Temperature stability. The enzyme (0.13 mg) in 500 μl of buffer I was preincubated for 15 min as well as 24 h at 10, 25, 32.5 40, 50, 60, and 70°C. The residual activity was determined at 25°C by adding 2.5 ml GPNA in buffer I (3.3 % methanol) giving a final con-

centration of 1 mM GPNA. Absorbance was measured after 15 min, as described under spectrophotometric assays.

Temperature optimum. 2.5 ml of 1.2 mM GPNA in buffer I (3.3 % methanol) were thermostated at 10, 25, 32.5, 40, 50, 60, and 70°C for 15 min. The reactions were started by adding 500 μl SGP3 (0.065 mg) in buffer I. Absorbance was measured at 410 nm.

pH-Stability and Ca^{2+}-dependence. 250 μl of the lyophilized enzyme (0.050 mg) in distilled water, with or without 0.03 M $CaCl_2$, was preincubated in 250 μl of 15 times diluted buffer II of pH 3.0, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0, 10.0, and 11.0 for 15 min as well as 24 h at 25°C, and tested for residual activity by adding 2.5 ml 1.2 mM GPNA in buffer II, pH 7.5 (3.3 % methanol). Absorbance at 410 nm was measured after 15 min at 25°C. The capacity of buffer II was good enough to ensure a pH of 7.5 ± 0.1.

pH-Optimum. 2.8 ml buffer II of pH 6.0, 7.0, 7.5, 8.0, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, and 12.0 were thermostated to 25°C and 100 μl GPNA in methanol added, giving a final concentration of 1 mM. The reaction was started by adding 100 μl of lyophilized SGP3 (0.050 mg) in 0.01 M $CaCl_2$ solution. Absorbance at 410 nm was measured after 15 min.

The effect of urea, methanol and acetone. 0.175 mg lyophilized SGP3 was incubated for 15 min at 25°C in 2.9 ml buffer II, pH 7.5, containing 0, 2, 4, 6 and 8 M urea with or without 0.03 M $CaCl_2$. The residual activity was determined at 25°C by adding 100 μl GPNA (final concentration 1 mM GPNA, 3.3 % methanol). The effect of various concentrations of acetone and methanol on the enzyme activity was determined towards 5 mM *N*-Ac-Ala_3ME as described under titrimetric assays.

Inhibition assays

Inhibition with ^{32}P-DFP was carried out by adding 25 μl of 10 mM ^{32}P-DFP dissolved in propyleneglycol to 250 μl of SGP3 (0.3 – 0.6 mg) or Pronase-P (2 mg) in buffer I. The degree of inhibition was studied by withdrawing 50 μl of the solution and testing for activity towards 3 ml of 1 mM GPNA solution as described below.

Inhibition with tosyl chloromethyl ketones was carried out according to the method of Shaw *et al.*[17,18] 2.9 ml buffer I with 1.67 % methanol, 5×10^{-5} M inhibitor and 5×10^{-7} M SGP3 was incubated at 25°C. After 20 h, 100 μl GPNA was added to give a final substrate concentration of 1 mM. The residual enzymatic activity was determined.

Inhibition with 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one was carried out essentially as described by Visser *et al.*[19] 5×10^{-5} M inhibitor and 5×10^{-7} M SGP3 in 2.9 ml 0.05 M phosphate buffer, pH 6.5, containing 10^{-4} M $CaCl_2$ and 5 % CH_3CN, was incubated for 10 h at 37°C. Residual enzymatic activity was determined in 1 mM GPNA, 3.3 % in methanol.

Inhibition with acetylprolylalanylprolylalanine chloromethyl ketone. 5×10^{-7} M enzyme in buffer II, pH 6.5, with a 100 fold molar excess of inhibitor, was incubated for 2 h at 37°C. Residual activity was determined with GPNA.

Enzyme assays

Titrimetric assays. Methyl and ethyl esterase activities were measured titrimetrically with an Autoburette ABU 12 and a Titrator TTT 1 c (Radiometer, Copenhagen). 2.95 ml of 0.1 M KCl-solution containing 2.5 % acetone and the substrate (1 – 20 mM) was added to the thermostated (25°C) reaction vessel. The pH was adjusted to 7.5 and 50 μl of enzyme in buffer I was added to start the reaction. 4.8 mM NaOH was used to maintain the pH at 7.5 and the measurements were carried out in an atmosphere of nitrogen. No significant adsorption of SGP3 to the electrodes was detected; *cf.* Ref. 10.

Spectrophotometric assays. All spectrophotometric determinations were corrected for blanks and were carried out in a Zeiss PMQ II spectrophotometer. *(a) Proteolytic activity* was measured according to the method of Kunitz [20] as described by Löfqvist and Sjöberg;[3] *(b) collagenolytic activity* was determined according to the method of Wünsch and Heidrich [21] but in buffer I at 25°C; *(c)* p*-nitrophenyl esterase activity.* The solubility of some of these esters is very low in water. A 0.030 M borate buffer, pH 7.5, containing 10 % acetone was therefore used to make 0.1 – 1 mM *p*-nitrophenyl ester solutions. The reaction was then started by adding 50 μl of the enzyme (0.115 – 59 μg) in buffer I to a

cuvette containing 2.95 ml of *p*-nitrophenyl ester solution. In order to minimize autolysis of the substrates, the reaction vessel was thermostated at 10°C. The liberated *p*-nitrophenol was assayed by measuring the optical density at 410 nm; *(d) Peptidase activity* towards BANA and GPNA was determined according to Erlanger *et al.*[22,23] 2.90 ml of 0.25 – 2 mM substrate in buffer I with 3.3 % methanol was incubated with 100 μl of SGP3 (10 μg), and the absorbance was monitored at 410 nm at 25°C. One GPNA-unit of the enzyme was defined as the amount of enzyme hydrolyzing 1 μmol GPNA per minute; (*e*) *Elastolytic activity* was determined by the congo-red elastin method according to Shotton;[24] (*f*) *Azocoll activity* was determined according to the method of Moore.[25] 60 mg azocoll was suspended in 16 ml of buffer I and constantly shaken at 25°C. The reaction was started by addition of 4 ml enzyme (0.004 – 0.4 mg) in buffer I. The activity was monitored by measuring the optical density at 520 nm (the absorption maximum of the product formed); (*g*) *Carboxypeptidase activity.* To 0.9 ml 10^{-4} M CBZ-Gly-Leu in buffer I was added 100 μl SGP3. Liberated Leu was assayed by the ninhydrin method;[26] (*h*) *Amidase activity.* 9.75 ml 1 mM substrate solution in buffer II with 5 % acetone was incubated with 250 μl SGP3 in buffer I. The reaction was carried out at 25°C. 2 ml samples were withdrawn at suitable time intervals and the enzyme was immediately inactivated by addition of NH_3-reagent as described by Balis.[27] The colour was fully developed after 2 h, and the absorbance was read at 640 nm; (*i*) *Activity towards β-naphthylamides* was determined according to the method of Goldbarg and Ruthenburg.[28] 2.90 ml of 0.25 – 2 mM L-Leu-β-naphthylamide in buffer I with 3.3 % methanol at 25°C, was incubated by addition of 100 μl of SGP3 (23 μg) in buffer I. Samples were withdrawn after 1 h and 20 h.

RESULTS

Isolation of the enzyme. Gel filtration of Pronase-P was carried out on a Sephadex G-75 column, and seven peaks (Fig. 1) with enzymatic activity were obtained. Peak G (approximately 300 ml) corresponds to the polyacrylamide

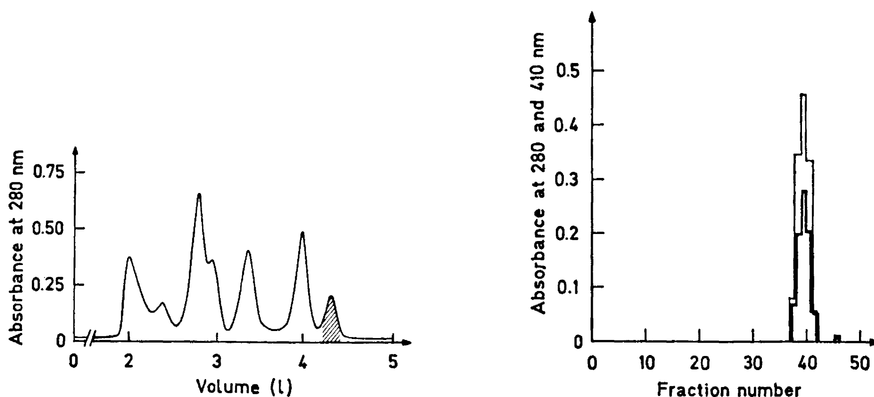

Fig. 1. Gelfiltration of Pronase-P on a Sephadex G-75 superfine column (10 × 90 cm). The peaks are designated A, B, C, D, E, F, and G, going from left to right. Fractions from G were pooled as indicated in the figure.

Fig. 2. Histogram showing enzymatic activities of peak G found in the different gel fractions of a small preparative polyacrylamide gel after 4 h electrophoresis (cathodic migration) in 0.38 M borate – 0.01 M Ca^{2+} buffer, pH 6.8. The gel fractions (1.5 mm thick) were extracted at pH 7.5 with a 0.03 M borate – 0.03 M $CaCl_2$-buffer, and tested for activity towards casein pH 7.5 (—) and GPNA pH 7.5 (–).

gel electrophoresis band 3, as shown by testing each fraction (25 ml) of the peak on analytical gels. At the same time evidence was gathered for homogeneity of the G-fractions. Homogeneous fractions were pooled as indicated in the figure and stored at 4°C.

Purity of the preparation. Further tests of purity were carried out with pooled enzyme concentrated 5 – 10 fold by ultrafiltration, in order to detect even very small amounts of impurities. *(a) Preparative polyacrylamide gel electrophoresis.* Proof of homogeneity was gathered by electrophoresis on small preparative gels. The activity of the eluates from the gel slices towards GPNA and casein pH 7.5 was tested. As can be seen from Fig. 2, there is one big peak only with almost coincident GPNA and casein activities from fraction to fraction, a very good criterion of the peak's homogeneity. Fraction 46 corresponds to about 1 % of the total activity towards casein and much less than 1 % of the total activity towards GPNA. *(b) Analytical polyacrylamide gel electrophoresis.* The purity of peak G was further assessed by analytical polyacrylamide gel electrophoresis in three different buffer media (see experimental procedure). Pooled SGP3 proved to be homogeneous in all three buffers. When concentrated enzyme was used it was possible to detect a slower moving impurity, estimated by dilution series to constitute about 1 %. No activity could be assigned to this band. A second contaminating band, similarly estimated to constitute about 1 %, moving faster than SGP3 could sometimes be detected. This faint band probably corresponds to the small impurity detected in fraction 46 of the preparative polyacrylamide gel.

After isolation regarding molecular size, SGP3 has thus been proved by charge-based separation to be about 98 % pure according to protein staining with coomassie blue. Based on catalytic activity the preparations have been found to be about 99 % pure. The 1 % enzymatic impurity detected in fraction 46 of the preparative gel most certainly originated in the F-peak, as it had the same migration position as a component from that peak. This impurity can thus be further minimized by more restrictive pooling. No contamination by aminopeptidases or carboxypeptidases could be detected, as no activity towards L-Leu-β-naphthylamide or CBZ-Gly-Leu could be demonstrated, even upon prolonged incubations.

Identity. The results of earlier investigations [1,4–6,9–11,14] lead to the conclusion that there are hardly any media in which Pronase can be dissolved without any change in its identity. A complete control of change in yield and specific activity of the enzyme throughout the separation and storage procedures is therefore a necessary pre-requisite for the characterization. The data in Table 1 give evidence for a high level of stability under the selected conditions. No loss in yield or activity could be indicated during the separation procedure. During storage at 4°C in buffer I, the loss of activity was about 0.3 % a day, as checked both by active site titration and activity towards GPNA. Table 1 also shows that the specific activity towards GPNA is constant throughout the separation procedure and storage, indicating that the loss in activity is followed by a proportional loss of active sites. Thus, no modified DFP-reacting enzyme molecules with altered catalytic activity seem to be generated. Furthermore, electrophoretic analysis of SGP3, which had been stored for six weeks, did not show increased amounts of stainable im-

Table 1. Yield and specific activity of SGP3 at isolation and storage.

Fraction	Amount of protein mg	Activity nmol GPNA · mg protein^{-1} min^{-1}	Total activity towards GPNA μmol GPNA min^{-1}	Yield (recovered GPNA-activity) %	^{32}P-DFP reactive enzyme nmol/mg protein	Specific activity nmol GPNA · nmol ^{32}P-DFP react. enz^{-1} min^{-1}
Pronase-P applied	1300	39.1	50.8			
Estimated values for SGP 3 in Pronase-P		31.3[b]	40.6	100	2.24[c]	14.0
Peak G	52.7[a]	782	41.2	101		
Pooled fractions (freshly prepared)	44.6[a]	782	34.9	86	55.5[c]	14.1
Pooled fractions (stored 30 days at 4°C)	44.6[a]	717	32.0	79	50.7[c]	14.1

[a] Calculated from E(1 %, 1 cm, 280 nm)=8.6 and the volume. [b] Calculated from our observation that peak G contains about 80 % of the activity of Pronase towards GPNA. [c] Calculated from the amount of ^{32}P-DFP incorporated into SGP3.

purities. This indicates that when autodigestion of an enzyme molecule once has started it seems to be followed by a rapid digestion into rather small fragments.

The charge of the active enzyme molecule also seems unaltered during the separation and storage procedures. Thus the mobility of SGP3 is the same when crude Pronase or freshly prepared or stored SGP3 are studied on analytical polyacrylamide gel electrophoresis.

As far as the identity of the enzyme has been studied during the separation and storage procedures, no change in charge, molecular size, or specific activity towards GPNA could be detected. Yield seems to be optimal, which also shows that digestion during the course of the separation is minimized.

Extinction coefficient

The extinction coefficient, E(1 %, 1 cm, 280 nm), was determined to 8.6, which is in quite good agreement with Wählby's value of 9,[9] while Gertler and Trop on the other hand report E(0.1 %, 1 cm, 280 nm) = 1.21.[10] Our absorption determination was made on fresh enzyme in NH_4HCO_3 solution, after which the weight was determined upon lyophilization. A 0.1 % solution of those amino acids, which absorb at 280 nm (in the same molar proportions as was found in the protein from amino acid analyses), gave an extinction coefficient of 0.86, which thus must be considered as a maximum value. Lyophilized enzyme was found to give non-reproducible and sometimes too high extinction coefficients. This is probably due to the formation of enzyme aggregates during lyophilization, causing light scattering and consequently too high absorption values and extinction coefficients. We could thus obtain reproducible E(1 %, 1 cm, 280 nm) values only when fresh enzyme, not lyophilized, was used for absorption determinations.

Calcium effect on electrophoretic mobility

The amount of Ca^{2+} in a lyophilized preparation of SGP3 was determined by atomic absorption on a Perkin-Elmer 303 and found to be less than 0.1 Ca-atoms per enzyme molecule. This preparation was used to demonstrate the relation between electrophoretic mobility and ionic strength of Ca^{2+} in buffers at constant pH (Table 2). The low mobility in Ca^{2+}-free solutions

Table 2. The electrophoretic mobility of SGP3 in 6 % polyacrylamide gels, 0.03 M borate buffer of various concentrations of Ca^{2+}.

Concentration of Ca^{2+} in the buffer (M)	Electrophoretic mobility (cm^2 sec^{-1} V^{-1}) × 10^6
10^{-6}	6
10^{-5}	7
10^{-4}	15
10^{-3}	25
10^{-2}	38

($[Ca^{2+}] \leq 10^{-5}$ M) indicates that the enzyme is only slightly positively charged at pH 6.8, with an IP just above 7. When more Ca^{2+} is added to the buffer, the mobility increases indicating that Ca^{2+} is bound to the enzyme, thus giving the molecule a more positive net charge. The enzyme does not seem to reach a point of saturation concerning Ca^{2+}, even at concentrations as high as 0.01 M.

Amino acid composition and molecular weight

Amino acid composition has been based on a molecular weight of 18 000 (see below), assuming 10.0 leucine residues. As can be seen from Table 3, there is excellent agreement between our data and the data of Johnson and Smillie.[11]

Table 3. Amino acid analyses of SGP3 and for comparison values from Johnson and Smillie [11] and Gertler and Trop.[10]

Amino acid residue	Number of amino acid residues per molecule SGP3 (assuming 10.0 Leu) according to: Bauer and Löfqvist 24 h hydrolysis	Bauer and Löfqvist Integral number	Johnson and Smillie [a]	Johnson and Smillie Integral number	Gertler and Trop 22 h hydrolysis	Gertler and Trop Integral number
Lys	traces	0	0.15	0	0.6 × 2.5 = 1.5	2
His	2.9	3	3.00	3	1.1 × 2.5 = 2.8	3
Arg	6.9	7	6.7	7[b]	2.8 × 2.5 = 7.0	7
Asp	16.1	16	15.9	16	7.0 × 2.5 = 17.5	18
Thr	21.7[c]	22	21.9[c]	22	8.1 × 2.5 = 20.3	20
Ser	20.2[c]	20	21.7[c]	22	8.5 × 2.5 = 21.3	21
Glu	8.0	8	8.2	8	3.6 × 2.5 = 9.0	9
Pro	3.9	4	4.4	4	2.0 × 2.5 = 5.0	5
Gly	31.4	31	31.7	32	12.8 × 2.5 = 32.0	32
Ala	18.7	19	18.9	19	8.1 × 2.5 = 20.3	20
1/2 Cys	3.7[d]	4	4.3[d]	4	1.5 × 2.5 = 3.8	4
Val	11.4	11	11.9[e]	12	5.1[f] × 2.5 = 12.8	13
Met	1.1[d]	1	1.0[d]	1	0.8 × 2.5 = 2.0	2
Ile	7.3	7	8.2[e]	8	3.1[f] × 2.5 = 7.8	8
Leu	10.0	10	10.0	10	4.0 × 2.5 = 10.0	10
Tyr	7.9	8	8.0	8	3.0 × 2.5 = 7.5	8
Phe	4.8	5	5.2	5	2.0 × 2.5 = 5.0	5
Trp	1.2[g]	1	0.61[h]	1	0.5[i] × 2.5 = 1.3	1

[a] Results are averages of duplicate hydrolyses at 24, 48, 72, and 96 h. [b] Corrected to 8 after partial amino acid sequence determination.[12] [c] Values are extrapolated to zero times of hydrolysis. [d] Values estimated after performic acid oxidation. [e] Average of values after 72 and 96 h. [f] Values after 48 h. [g] Estimated by the method of Edelhoch.[16] [h] Estimated by the method of Harrison and Hofman and calculated relative to the arginine value.[11] [i] Hydrolysed in the presence of 4 % thiodiglycolic acid.[10]

The difference in serine residues is probably due to the use of a wrong correction factor, this amino acid being partly hydrolyzed after 24 h.[29] Our slightly low valine and isoleucine values are certainly due to the short hydrolysis time.[29]

"Enzyme II", one of the "pronase elastases", studied by Gertler and Trop [10] is apparently identical to SGP3, as mentioned in the introduction. The reported number of amino acid residues of "Enzyme II" was, however, very low (Table 3), since it was based on a molecular weight of 7 000 and 4.0 leucine residues. If these values are multiplied by 2.5 (which gives 10.0 leucine residues and a molecular weight around 17 500) it can be seen that there is quite good agreement between the new values for "Enzyme II", our values, and those obtained by Johnson and Smillie.

Wählby [9] has shown that one ^{32}P-DFP-molecule reacts with one molecule of enzyme. The molecular weight can thus also be calculated from the number of ^{32}P-DFP molecules, which react with a known amount of 100 % active enzyme. As can be seen in Table 1, it has been possible to use this method, and the molecular weight for SGP3 was estimated to 18 000. Molecular weight calculations, based on our amino acid analyses data, give a value around 17 750.

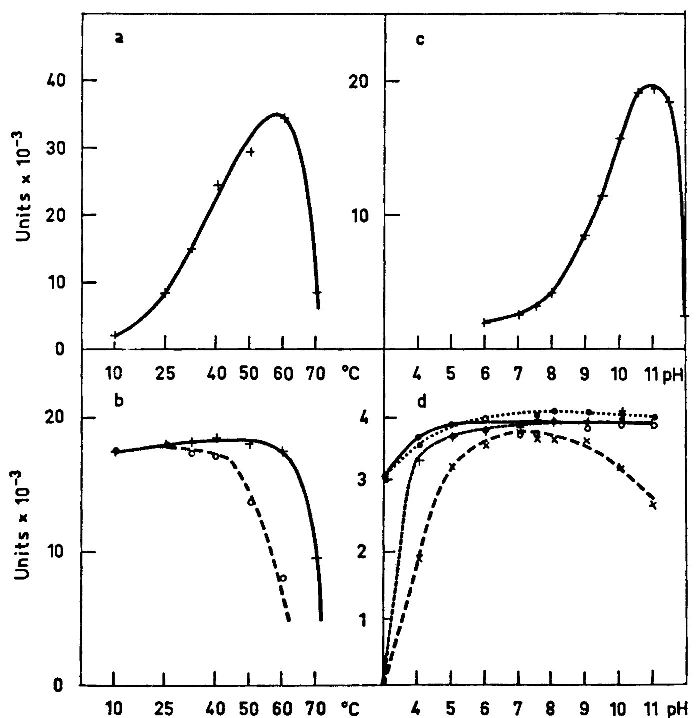

Fig. 3. (a) *Temperature optimum* after 15 min incubation at pH 7.5, (b) *temperature stability* after 15 min incubation (+) and 24 h incubation (O) at pH 7.5, (c) *pH-optimum* after 15 min incubation at 25°C, and (d) *pH-stability and* Ca^{2+}*-dependence* after 15 min (O) and 24 h (×) without Ca^{2+}, and 15 min (O) and 24 h (+) with Ca^{2+} at 25°C. Substrate: 1 mM GPNA.

Optimal conditions and stability of the enzyme

As seen in Fig. 3b, at temperatures over 40°C (pH 7.5) the enzyme is rather unstable even in Ca^{2+}-containing buffers when incubated for several hours. Temperature optimum (Fig. 3a) has been estimated to 57°C, for an incubation time of 15 min. The enzyme is stable in the range pH 5.0 – 11.0 (Fig. 3d) when incubated in buffers containing 3×10^{-2} M Ca^{2+}, but without Ca^{2+} it is stable only in the range around pH 6 – 9. The stability declines abruptly below pH 4, without Ca^{2+} already at pH 5, reaching zero at pH 3, where the enzyme is irreversibly denatured. To minimize the inactivation it is thus recommended to store the enzyme in a buffer 0.03 M in Ca^{2+} even at 4°C.

The pH-optimum of SGP3 (Fig. 3c), when GPNA is used as substrate (15 min assay), is found between pH 10 and 11, where the enzyme is about six times as active as at the commonly used pH 7.5.

The effects of acetone and methanol on the activity of SGP3 towards the watersoluble substrate *N*-Ac-Ala_3-ME have briefly been checked. Addition of 2.5 % acetone or methanol caused a decrease of 40 – 50 % in activity and 5 % gave a decrease of 60 – 65 %.

Upon incubation with urea, the activity of the enzyme rapidly decreases (Fig. 4). In 8 M urea the activity is only about 5 % of the original. The residual activities after urea treatment have been measured with GPNA in 3.3 %

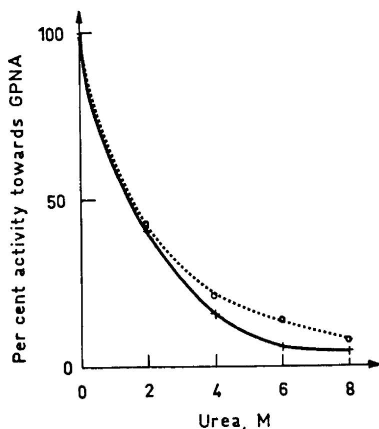

Fig. 4. Influence of urea on the activity of the enzyme as measured with 1 mM GPNA as substrate (3.3 % methanol). (O) with 0.03 M Ca^{2+}, (+) without Ca^{2+} (lyophilized).

methanol. Since methanol causes decreases in the activity of SGP3, it cannot be outruled that the effect of urea is enhanced by the presence of methanol. Nevertheless the decrease in activity is so rapid, that the enzyme must be considered to be rather susceptible towards urea.

Inhibitor specificity

Enzyme activity towards GPNA rapidly decreases on incubation with a 20-fold molar excess of DFP, showing that SGP3 is a serine enzyme as earlier pointed out by Wählby,[4] and Gertler and Trop.[10] The results of the inhibition

Table 4. Inhibition of enzyme activity by tosyl chloromethyl ketones. The results are given in percent inhibition of the activity towards GPNA after 20 h incubation at pH 7.5 and 25°C. The molar ratio of inhibitor to enzyme was 100:1.

Inhibitor	Inhibition (%)
T Ala CK	0
T Val CK	5
T Leu CK	15
T Phe CK	20
T Lys CK	0

studies with various tosyl chloromethyl ketones are presented in Table 4. TPheCK, normally considered as a site-specific chymotrypsin inhibitor,[30] is the most potent of these inhibitors, but TLeuCK and, to a lesser extent, T ValCK also inhibit the enzyme.

Two compounds known as powerful elastase inhibitors have also been tested. One of them, 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one, caused, when in 100-fold molar excess over SGP3, only about 5 % inhibition (10 h, 37°C, pH 6.5). Under similar conditions elastase was inhibited to 90 %.[19] The site of inhibition in SGP3 has not been determined, but in elastase the γ-carboxyl of Glu-6 is alkylated.[19] The other inhibitor, Ac-Pro-Ala-Pro-Ala CK, made as a specific inhibitor for elastase (Professor Blout, personal communication), caused an inhibition of 10 % of SGP3 activity towards GPNA, after 2 h (37°C, pH 6.5) with a 100-fold molar excess of inhibitor.

Substrate specificity

1. Specificity towards some natural and some synthetic substrates. As can be seen in Table 5 the enzyme is about equally active towards casein both at pH 7.5 and pH 10.0. Further, SGP3 is active towards congo-red elastin. Though no unit of activity is defined for congo-red elastin,[24] the activity of SGP3 towards this substrate is found to be 10 – 20 % of that of pure elastase.[24] This is in good agreement with the data of Gertler and Trop.[10]

SGP3 is also active towards the synthetic collagenase A substrate, as well as towards azocoll. GPNA, which is a chymotrypsin substrate,[23] is also a good

Table 5. Activity towards some natural and some synthetic substrates at 25°C.

Substrate	Activity
Casein, pH 7.5	0.20 OD_{280} units mg enz.$^{-1}$ min^{-1}
Casein, pH 10.0	0.21 OD_{280} units mg enz.$^{-1}$ min^{-1}
Azocoll	4.0 OD_{520} units mg enz.$^{-1}$ min^{-1}
Collagenase A substrate	6.7×10^{-2} μmol mg enz.$^{-1}$ min^{-1}

substrate for SGP3 and has been used in many of the assays throughout this investigation, as it is a stable and easily measureable substrate. The kinetic constants for SGP3 using GPNA as substrate are $K_m = 0.91 \times 10^{-3}$ M, $k_{cat} = 0.22$ sec^{-1} and $k_{cat}/K_m = 240$ sec^{-1} M^{-1} (2 % methanol, pH 7.5), whereas the constants for chymotrypsin under similar conditions are $K_m = 2.8 \times 10^{-4}$ M, $k_{cat} = 0.013$ sec^{-1}, and $k_{cat}/K_m = 46.4$ sec^{-1} M^{-1}.[23] Thus the K_m for chymotrypsin is about 3 times lower, k_{cat} is about 17 times lower and k_{cat}/K_m about 5 times lower than the corresponding constants for SGP3. Thus, SGP3 is more efficient in cleaving GPNA. Methanol (3.3 %) increased K_m 1.5 times, but had little effect on k_{cat} ($K_m = 1.4 \times 10^{-3}$ M, $k_{cat} = 0.24$ sec^{-1} and $k_{cat}/K_m = 170$ sec^{-1} M^{-1}).

Interestingly, no activity towards L-Glu-L-Phe-β-naphthylamide, the β-naphthylamide analogue of GPNA, could be detected even upon 24 h incubation, indicating that the naphthylamide group is too bulky to permit binding.

2. *Specificity towards methyl and ethyl esters.* ATEE, a chymotrypsin substrate, is the most readily hydrolyzed of these esters, as can be seen in Table 6. SGP3 also shows a little activity towards the trypsin substrate BAME, but even on prolonged incubations it is not at all active towards BANA, another trypsin substrate.[22]

Table 6. Activity of SGP3 towards some methyl and ethyl esters, at pH 7.5 in 0.1 M KCl (2.5 % in acetone) at 25°C.

Substrate	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})
ATEE	360	2800
N-Ac-Ala ME	2	15
N-Ac-Ala_2 ME	10	450
N-Ac-Ala_3 ME	70	4400
N-Benzoyl-Ala ME	1	100
BAME	0.8	250

Activity towards the *N*-benzoyl methylesters of hydrophobic amino acids could not be determined at acceptable acetone concentrations, since the solubilities of some of these compounds are quite low, and the enzyme is most sensitive towards even low concentrations of acetone. It was, however, possible to use the glycine and alanine compounds, but SGP3 was active only towards the latter.

3. *Specificity towards* p-*nitrophenyl esters.* The low solubility and the high rate of self-hydrolysis of these esters makes it difficult to obtain accurate determinations of K_m and k_{cat}. Thus our values presented in Table 7 can be regarded as only approximate, though within the right order of magnitude. As can be seen, SGP3 is characterized by very high k_{cat}/K_m values for the Leu and Tyr analogues, like α-chymotrypsin, but in contrast to elastase. The figures for the Gly and Ala compounds are, however, similar to those determined for elastase. Interestingly, the k_{cat}/K_m values of SGP3 and

Table 7. Comparison of k_{cat}/K_m values for SGP3, alkaline protease C, elastase, and chymotrypsin towards some *p*-NPE compounds. The activity of SGP3 was determined at 10°C in 0.015 M borate buffer, pH 7.5, containing 10 % acetone.

p-NPE compound	SGP3 (Bauer and Löfqvist)		Alkaline protease C(=SGP1) [a]	Elastase [b,c]	α-Chymotrypsin [b]
	$k_{cat}(s^{-1})$	$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}/K_m(s^{-1}M^{-1})$
CBZ-Gly	5	10^4	1.8×10^4	1.5×10^4 [b]	1.9×10^5
CBZ-Ala	20	2×10^5	—	1.85×10^5 [c]	—
CBZ-Leu	15	4×10^6	2.7×10^6	2.9×10^4 [b]	3.45×10^6
CBZ-Tyr	500	5×10^7	1.25×10^7	80 [b]	11×10^7
CBZ-Pro	0.06	200	—	20 [c]	—
Ac	0.04	300	—	470 [b]	

[a] All assays were conducted at 25°C in phosphate buffer ($I=0.05$, pH 7.5), containing 1.66 % acetonitrile.[31] [b] All assays were conducted at 25°C in 0.1 M phosphate buffer (pH 7.8).[32] [c] All assays were conducted at 25°C in phosphate buffer ($I=0.1$).[33]

Table 8. Activity towards amide substrates. Activity is given as % hydrolysis of the amide bond after 72 h at 25°C, pH 7.5 (5 % acetone).

Substrate	% hydrolysis
N-Ac-L-Ala·NH_2	0
N-CBZ-Gly-L-Leu·NH_2	2
N-CBZ-Gly-L-Phe·NH_2	9
N-CBZ-Gly-L-Tyr·NH_2	12

Narahashi's "alkaline protease C" (=SGP1),[31] reveal very little, if any, difference in specificity as concerns the Gly, Leu, and Tyr analogues.

4. Specificity towards amide substrates. As can be seen from Table 8 the amidase activity of the enzyme is very low. Interestingly, the enzyme is more active even towards the phenylalanine than the leucine analogue. Like elastase, this enzyme does not appear to cleave N-acetyl-L-Ala amide. Although Thompson and Blout [34] showed this substance to be a competitive inhibitor for elastase, it did not detectably inhibit SGP3.

DISCUSSION

In this investigation, special attention has been drawn to the stability of enzyme SGP3 during the purification and storage procedures. This is justified by the fact that Pronase-P, the starting material, is a very complex mixture of active proteolytic enzymes, which may cause a number of active and inactive degradation products when subjected to purification. In addition, several of the enzymes in Pronase show amidase activity (Löfqvist, B., unpublished) and may thus cause deamidation reactions. This could *e.g.* be the case in the investigation by Johnson and Smillie.[11] They obtained two proteolytically active peaks on Bio-Rex 70, with identical amino acid compositions but with different specific activities – one (SGPA1) showing only half the specific activity of the other (SGPA2) towards ATEE. Obviously, SGPA1 and SGPA2 differed in charge since they were not equally retarded on the ion exchanger, and Johnson and Smillie also suggested (based on their sequence work) that SGPA1 may be generated from SGPA2 by deamidation of Gln 192 A. If so, this could very well explain the differences both in charge and specific activity, since Gln 192 A ought to be located in the vicinity of the active Ser 195. In this connection it is also interesting to note that elastase has a Gln in position 192 located in the active region of that enzyme.[35]

It is thus evident that care must be taken during the isolation procedure and storage so that no changes of the enzyme identity will occur, and this should thoroughly be checked. As shown in Table 1 it has been possible to accomplish such mild conditions in our separation, as the specific activity is unaltered.

To test the homogeneity of SGP3 we also tried the Bio-Rex 70 separation method of Johnson and Smillie.[11] It was not possible, however, to elute our

SGP3 preparation under the prescribed conditions, but the enzyme was released when a buffer of a higher pH and a higher ionic strength was used. This indicates that our enzyme preparation should have a higher isoelectric point than SGPA2. Both separations were performed in Ca^{2+}-free buffers, where we have shown that no Ca^{2+}, which could alter the net charge, was bound to the enzyme. Thus, the results suggest that our enzyme preparation should be less deamidated than SGPA2. Analytical polyacrylamide gel electrophoresis of Pronase-P and stored SGP3 also verify that no change in charge and thus no deamidation has occurred at our conditions of isolation and storage.

During storage of SGP3 a loss in total activity, but not in specific activity, is seen (Table 1). As discussed above, deamidation reactions cannot be responsible for this loss in activity, and accordingly the enzyme is exposed to another type of denaturation causing loss of enzyme. Analytical polyacrylamide gel electrophoresis does not reveal any new stainable components, and K_{av} for SGP3 on Sephadex G-75 is not influenced by storage.[13] These facts indicate that the loss in activity is caused by autodigestion of the enzyme into rather small fragments, and that no high molecular intermediates are accumulated during this process. Such a digestion also explains the parallel losses in active sites and amount of enzyme. It is also in good agreement with the findings of Klevhag and Löfqvist,[13] who showed that several of the Pronase-enzymes, including SGP3, were digested into low molecular components in the absence of Ca^{2+} or by prolonged storage at neutral pH. We thus conclude that even if it has not been possible to avoid a certain loss in yield and active sites during our isolation and storage procedures, this has not caused any change in the identity of SGP3.

A third observation concerning the identity of SGP3, which, however, does not overthrow the statements above, may be worth mentioning in this context. Lyophilized preparations of SGP3 sometimes show heterogeneity on polyacrylamide gel electrophoresis. The additional bands are slower migrating than SGP3. They are all active towards casein and GNPA, and the ratio of activity between casein and GPNA for all these components are equal to that of homogeneous SGP3. One possible explanation to the heterogeneity is that lyophilization sometimes may cause the formation of polymeric forms of SGP3.

Some properties of SGP3 in relation to earlier investigations on this enzyme and chymotrypsin and elastase. Molecular weight calculations, based on our amino acid analyses data, give a value around 17 750 for SGP3, while Johnson and Smillie[11] similarly derived a molecular weight of 18 276. The latter value is probably the more accurate, since the former is based on results known to be low in some cases. Our determination of molecular weight with ^{32}P-DFP, also gave a value of 18 000. Wählby determined, however, a molecular weight of approximately 16 000 by using an ultracentrifugation technique,[9] but a value of 18 000 can possibly be within the limits of error for the technique used. Estimations of molecular weight by gel filtration on Sephadex have given still lower values. Klevhag and Löfqvist[13] estimated a molecular weight of 15 000 on Sephadex G-75, and Awad *et al.*[8] similarly derived 15 500 on Sephadex G-100. These somewhat lower values might possibly indicate a slight interaction between Sephadex and the protein. The extremely low value of

7 000 obtained by Gertler and Trop by gel filtration on Sephadex G-75 must be wrong, and is probably due to an artifact, as they also suggest themselves.[10]

The pH stability curve of SGP3 (in Ca^{2+}-buffers) shows that while it is stable between pH 5.0 and 11.0, it is irreversibly denatured at pH 3 (Fig. 3d). This behaviour is in contrast to that of chymotrypsin and trypsin, but similar to that of elastase.[24]

The pH-optimum curve for SGP3 (Figure 3c), with an optimum far on the alkaline side, indicates the dissociation of a functional group around pH 9 and 10.

The effects of urea and methanol and acetone show that SGP3 is most sensitive to the polarity and to the hydrogen bonding capacities of the environment, thus indicating a rather loose tertiary structure.

Earlier inhibition studies with TPheCK have shown both distinctly higher [10] and distinctly lower [11] inhibitions than we have found. These results are not necessarily inconsistent, however, since the excess of the inhibitor and the incubation pH and time have not been the same. The fact that SGP3 is inhibited by certain tosyl chloromethyl ketones (Table 4) might indicate that a histidine residue, necessary for activity, is present in the active site of SGP3, as has been proved for chymotrypsin with TPheCK,[17] and for trypsin with TLysCK.[18] TPheCK, normally considered to be a site-specific inhibitor for chymotrypsin,[30] only partly inhibits SGP3, indicating that the subsites of this enzyme differ from those of chymotrypsin. The low inhibition of SGP3 with inhibitors site-specific for elastase, suggests that the subsites of SGP3 also differ from those of elastase.

From the substrate specificity data (Tables 5 – 8), it can be seen that SGP3 shows a broad substrate specificity, and is highly active towards both typical chymotrypsin substrates (ATEE, CBZ-Tyr-*p*NPE) and typical elastase substrates (*N*-Ac-Ala_3ME and congo-red elastin). Like elastase, SGP3 shows high activity towards *N*-Ac-Ala_3ME, less towards *N*-Ac-Ala_2ME, and low activity towards *N*-Ac-AlaME. This might indicate an extended active center, as postulated for elastase by Thompson and Blout.[34]

The facts that SGP3 has an alkaline pH-optimum, that it is totally inhibited by DFP, and that it is proteolytically active, permit its classification as an alkaline serine protease.

Relation of SGP3 to some other microbial serine proteases. The relation of SGP3 to other microbial serine proteases, especially to those in Pronase, is interesting. Though three "elastase-like enzymes" from Pronase have been reported,[10] only two of these, the low molecular weight ones, seem to be closely related, with a sequence around their active seryl residues similar to that of chymotrypsin.[5] The third "elastase-like enzyme", which is of a higher molecular weight, has recently been shown to have an amino acid sequence around its active seryl residue similar to that of subtilisin.[8] What differences could there be between the two low molecular weight enzymes? Table 7 only possibly indicates faint differences in specificity. Preliminary results from our laboratory show, however, that SGP3 has a much higher specific activity towards GPNA than SGP1. Further differences are indicated by Gertler and Trop, who showed that their "Enzyme II" (= SGP3) reacted more rapidly with ATEE than with *N*-Ac-Ala_3ME, whereas the reverse was true for "Enzyme

III" (=SGP1).[10] Careful comparative investigations with both SGP1 and SGP3 must evidently be undertaken in order to detect further differences in specificity between these two enzymes.

SGP3 is, as mentioned above, rather susceptible towards urea. This is in marked contrast to the serine enzyme (identical to SGP1) studied by Siegel *et al.*,[36] which was unusually stable in 6 M quanidinium chloride and in 8 M urea. That enzyme also proved to be markedly stabilized by Ca^{2+}. As seen from Fig. 4 this is evidently not the case for SGP3. These facts indicate that there could be important structural differences between the two enzymes.

It might be argued that SGP3 is derived from SGP1 by limited digestion, since the latter enzyme seems to have slightly higher molecular weight. Preliminary amino acid analysis of SGP1 from this laboratory, do not verify such a hypothesis, as SGP1 seems to have, for example, fewer Glu/Gln than SGP3.

Interestingly, SGP3 does also appear to have some properties in common with the well-studied α-lytic protease from *Sorangium sp.*[37,38] Thus SGP3, α-lytic protease and elastase are "low-lysine, high-arginine" enzymes,[38] in contrast to chymotrypsin and trypsin. This make the first-mentioned enzymes subject to less losses of net charge, when the pH is increased from 8 to 10. Further, α-lytic protease seems to be quite similar to elastase in specificity, at least towards certain small synthetic substrates. Unlike SGP3, however, it does not hydrolyze ATEE, and is not at all inhibited by tosyl chloromethyl ketones. As stated by Kaplan and Whitaker,[37] "the substrate binding properties of the α-enzyme are appreciably different from those of chymotrypsin". Evidently the α-enzyme also differs from SGP3 in these properties.

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