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Insulin-like Activity of Proteases. VI.[1] Purification and Some Properties of an Acid-stable *N*-Succinyl-trialanine *p*-Nitroanilide-hydrolyzing Protease from Pronase

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An acid-stable *N*-succinyl-L-trialanine *p*-nitroanilide-hydrolyzing protease was purified from Pronase E with a protein yield of 6% by affinity chromatography on arginine peptides-Sepharose or soybean trypsin inhibitor-Sepharose. The enzyme showed a potent increasing effect on the glycogen content in an isolated mouse diaphragm. It was homogeneous both in disc and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, had an optimum pH of 8.5, and was stable in the pH range from 2 to 10, whereas its activity was lost at pH 12. Its molecular weight was estimated to be 20800. The sequence up to thirty-two residues from the N-terminus was determined with guanidine-treated and *S*-carboxymethylated enzymes. The N- and C-terminal residues were isoleucine and tyrosine, respectively. The results suggest that this enzyme is mainly responsible for the insulin-like activity of Pronase and that it is homologous with Protease B which was purified from Pronase B (L. Jurášek, M.R. Carpenter, L.B. Smille, A. Gertler, S. Levy, and L.H. Ericsson, *Biochem. Biophys. Res. Commun.*, **61**, 1095 (1974)), though some differences were found between the C-terminal regions of the two enzymes.

Keywords——acid-stable protease; Pronase; affinity chromatography; insulin-like activity; glycogen-increasing effect; mouse diaphragm

Several proteases mimic the *in vitro* actions of insulin on rat diaphragms, fat cells, and frog sartorius muscles.[2-5] It has been considered in general that the insulin-like effects of proteases are partially due to digestion of the cell surface at a limited region. We have previously reported that acid-treated Pronase which had lost more than 95% of its original caseinolytic activity still showed the insulin-like activity[6] and that Pronase lost its insulin-like activity on isolated mouse hemidiaphragms after treatment with diisopropylphosphofluoridate (DFP) but not with 1-chloro-3-tosylamide-7-amino-2-heptanone (TLCK) or L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK).[7] We have partially purified a protease having a potent glycogen-increasing effect on the hemidiaphragms from Pronase and have shown that the enzyme hydrolyzes *N*-succinyl-L-trialanine *p*-nitroalinide (Suc-$(Ala)_3$-*p*NA) at a relatively high rate.[7] These results suggest that Pronase contains an acid-stable serine protease possessing insulin-like activity.

The purpose of the present work was to purify this enzyme from Pronase and to clarify its enzymatic and chemical properties.

Experimental

Animals——Male dd-Y mice weighing about 20 g were fed a pellet diet (CE-2, Clea Japan Co., Tokyo) for at least three days and fasted for 18 to 20 h before use.

Chemicals——Pronase (from *Streptomyces griseus*, type E, 70 PUK/mg) was a gift from Kaken Chemical Co., Tokyo. Insulin (from bovine pancreas, 24 IU/mg), carboxypeptidase A (from bovine pancreas, type I), *acetyl-L-trialanine methyl ester* (Ac-$(Ala)_3$-OMe), and soybean trypsin inhibitor (SBTI, type I-S) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Carboxypeptidase C_{ua} was purified from the exocarp

of *Citrus unshiu* Marc.[8] Amyloglucosidase (1,4-α-D-glucan glucohydrolase, from *Aspergillus niger*) was obtained from Boehringer Mannheim Yamanouchi Co., Tokyo. Protamine sulfate was a product of Nakarai Chemicals Co., Kyoto. Glucose AR-II reagent and sequenator reagents (sequanal grade) were purchased from Wako Pure Chemicals Industries, Osaka. Sepharose 4B was obtained from Pharmacia Fine Chemicals Co., Uppsala, Sweden. Benzoyl-L-arginine *p*-nitroanilide HCl (Bz-Arg-*p*NA), L-leucine *p*-nitroanilide (Leu-*p*NA), carbobenzoxy-glycyl-L-leucine (z-Gly-Leu), and Suc-$(Ala)_3$-*p*NA were purchased from the Protein Research Foundation, Osaka. Glutaryl-L-phenylalanine *p*-nitroanilide (Gl-Phe-*p*NA) and TLC aluminum sheets (silica gel 60 F_{254}) were obtained from E. Merck, Darmstadt, West Germany. Other chemicals used were of analytical grade.

Conjugation of Arginine Peptides (AP) and SBTI to Sepharose——AP-Sepharose was prepared by the method of Yokosawa *et al.*[9] A mixture of 2.5 g of protamine sulfate and 10 mg of TPCK-treated trypsin[10] was incubated in 75 ml of 0.1 M phosphate buffer, pH 8.0, at 37°C for 100 min. The digestion was stopped by immersing the reaction mixture in boiling water for 30 min. After centrifugation, the supernatant containing AP was adjusted to pH 9.0 and used for the coupling reaction. Coupling to Sepharose 4B was carried out by the cyanogen bromide method of Cuatrecasas.[11] SBTI-Sepharose was prepared by the method of Feinstein.[12]

Determination of Enzymatic Activities——The hydrolytic activities towards Suc-$(Ala)_3$-*p*NA and Leu-*p*NA were determined in 0.05 M Tris-HCl buffer, pH 8.5.[7,13] The rates of hydrolysis of Bz-Arg-*p*NA, Gl-Phe-*p*NA, and z-Gly-Leu were determined in 0.05 M Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$.[7,14] Esterolytic activity towards Ac-$(Ala)_3$-OMe was determined in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl at 30°C by a colorimetric method.[15] One unit of enzymatic activity is defined as the amount of enzyme which forms 1 μmol of product per min under the assay conditions. Specific activity is expressed as unit/mg of protein.

Determination of Glycogen-increasing Effect on Isolated Mouse Diaphragm——An isolated mouse diaphragm was incubated with samples in 3.1 ml of Krebs-Ringer bicarbonate buffer, pH 7.9, containing 5 mM glucose under an atmosphere of 95% O_2–5% CO_2 with shaking at 120 strokes/min at 37°C for 45 min. Glycogen was separated from the treated diaphragm by the method described in a previous paper[16] and assayed by the enzymatic method using amyloglucosidase and glucose AR-II reagent.[17]

Gel Electrophoresis——Disc electrophoresis was performed in 0.13 M acetate buffer, pH 4.5, using 7% polyacrylamide gel, according to the method of Reisfield *et al.*[18] For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the enzyme was treated with 8 M guanidine HCl (pH 2.5) at 0°C for 4 h then dialyzed against deionized water. Electrophoresis was carried out in 0.1 M phosphate buffer, pH 7.2, using 10% polyacrylamide gel containing 0.1% SDS.[19]

Amino Acid Analysis——The enzyme (1 mg) was hydrolyzed with 0.5 ml of 6 N HCl under a vacuum in a sealed Pyrex glass tube at 110°C for 12, 24, 48, and 72 h. After removal of the HCl under reduced pressure, the hydrolysates were dissolved separately in 3 ml of 0.2 M citrate buffer, pH 2.2, and the amino acid contents were determined with a Hitachi KLA-5 amino acid analyzer. The content of tryptophan was determined on separate, unhydrolyzed samples by the ultraviolet spectrophotometric method.[20] The content of half-cystine was determined on an 18-h hydrolysate after conversion of cystine to cysteic acid by oxidation with performic acid.[21]

Carbohydrate Analysis——Carbohydrates were qualitatively identified by thin layer chromatography on silica gel plates after hydrolysis of the enzyme in 3 N HCl at 110°C for 2 h under a vacuum.[22]

Determination of Terminal Sequence——The N-terminal sequence of guanidine-treated and *S*-carboxymethylated enzymes was determined with a JEOL JAS-47K sequence analyzer, essentially according to the method of Edman and Begg.[23] The latter enzyme was prepared by alkylation with monoiodoacetic acid after reductive cleavage of cystine with dithiothreitol.[24] Samples (98—157 nmol) dissolved in 50% acetic acid were fixed on the wall of a spinning cup and coupled with phenyl isothiocyanate in 0.5 M Quadrol buffer, pH 9.5. The amino-terminal residue was released by addition of anhydrous heptafluorobutyric acid and extracted with *l*-chlorobutane. The phenyl thiazolinone derivatives were converted to the phenyl thiohydantoin derivatives by treatment with 1 N HCl at 80°C for 10 min. These derivatives were determined with a high performance liquid chromatograph equipped with a μBondapack C_{18} column (0.4 × 30 cm, Nihon Waters Ltd., Tokyo), eluting stepwise with 0.01 M ammonium acetate and a solvent system of methanol–acetonitrile–H_2O (8: 1: 1). Identification was also carried out by thin layer chromatography with a solvent system of *n*-heptane–propionic acid–1,2-dichloroethane (58: 17: 25) on TLC aluminum sheets (silica gel 60 F_{254}). Phenyl thiohydantoin-serine and -threonine were identified as alanine and α-aminobutyric acid, respectively, by thin layer chromatography on silica gel plates after hydrolysis in 6 N HCl at 105°C for 24 h under a vacuum.

For analysis of the C-terminal region, guanidine-treated enzyme (1 mg) was digested with DFP-treated carboxypeptidase A (100 μg) in 0.1 M phosphate buffer, pH 7.5,[25] and with carboxypeptidase C_{ua} (100 μg) in 0.1 M citrate buffer, pH 5.5.[8] The digestion was stopped by the addition of 50% trichloroacetic acid at various times. After centrifugation of the solutions, the concentrations of amino acids released were determined with the amino acid analyzer.

Results

Purification

Pronase (100 mg) was applied to a 2×17 cm column of AP–Sepharose and eluted with 0.05 M Tris-HCl buffer, pH 7.5, and then with 5 mM HCl (Fig. 1). Three peaks of absorbance at 280 nm were found. The hydrolytic activities towards Suc–$(Ala)_3$–*p*NA and Gl–Phe–*p*NA were found in the fraction (AP-1) behind the first protein peak. Most of the activity towards Bz–Arg–*p*NA, on the other hand, was concentrated in the last protein fraction. Rechromatography of AP-1 was carried out by the same procedure to eliminate completely the trypsin-like enzyme remaining in it. The fraction (AP-2) which was eluted with 0.05 M Tris-HCl buffer, pH 7.5, was subjected to affinity chromatography on SBTI-Sepharose (2×20 cm) with the successive use of 0.05 M Tris-HCl buffer, pH 8.5, and 5 mM HCl as eluents. The fraction (SBTI-1) eluted with 5 mM HCl was adjusted to pH 1.5 with 0.2 N HCl and allowed to stand at 4°C for 15 min. The HCl-treated fraction was readjusted to pH 8.5, applied to a column of SBTI–Sepharose, and eluted stepwise with 0.05 M Tris-HCl buffer, pH 8.5, H_2O, and 1 mM HCl (Fig. 2). The profile of protein eluted with 1 mM HCl paralleled that of the activity towards Suc–$(Ala)_3$–*p*NA. No activity towards either substrate was found in the other fractions. The active fraction was lyophilized without dialysis. The enzyme obtained is hereafter called Suc–$(Ala)_3$–*p*NA-hydrolyzing protease (STA-protease). The protein yields of the various steps were 56 (AP-1), 43 (AP-2), 11 (SBTI-1), and 6% (STA-protease).

Table I compares the hydrolytic activities of STA-protease and other fractions from Pronase towards various substrates. Pronase itself showed hydrolytic activities towards all the substrates. SBTI-1 hydrolyzed Suc–$(Ala)_3$–*p*NA at the highest rate among the fractions tested, presumably because this fraction contains a protease(s) hydrolyzing the substrate at a higher rate than STA-protease and being unstable to the HCl-treatment. STA-protease also

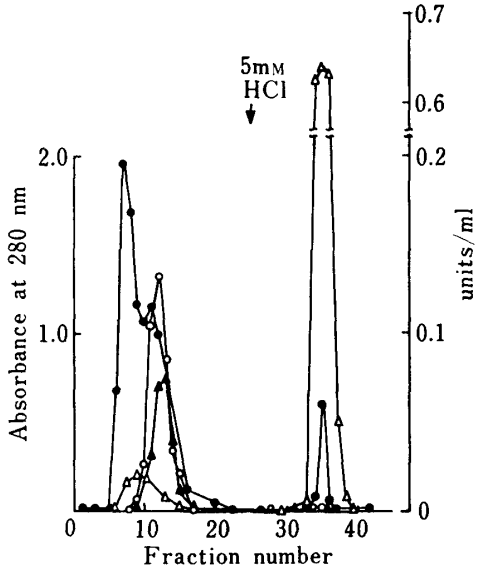

Fig. 1. Affinity Chromatography of Pronase on AP-Sepharose

Pronase (100 mg) was applied to a 2×17 cm column of AP-Sepharose and eluted with 0.05 M Tris-HCl buffer, pH 7.5, and then with 5 mM HCl. The flow rate was 150 ml/h and the effluent was collected in 10-ml fractions. The arrow indicates replacement of the buffer with 5 mM HCl. Profiles show the absorbance at 280 nm (●) and the hydrolytic activities towards Suc–$(Ala)_3$–*p*NA (○), Gl–Phe–*p*NA (▲), and Bz–Arg–*p*NA (△).

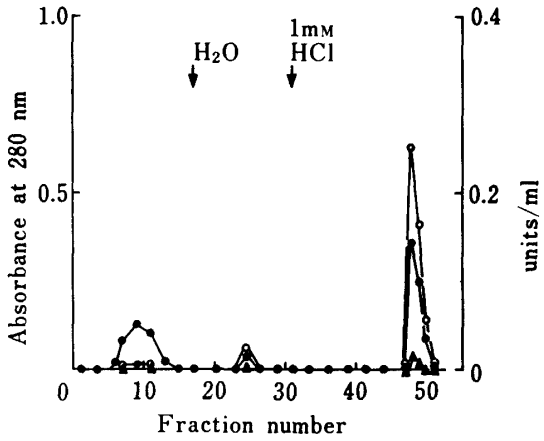

Fig. 2. Affinity Chromatography of HCl-treated SBTI-1 on SBTI-Sepharose

The HCl-treated SBTI-1 fraction (30 ml) was applied to a 2×20 cm column of SBTI-Sepharose and eluted stepwise with 0.05 M Tris-HCl buffer, pH 8.5, H_2O, and 1 mM HCl. The flow rate was 150 ml/h and the effluent was collected in 10-ml fractions. The arrows show replacement with H_2O and 1 mM HCl. Profiles show absorbance at 280 nm (●) and the hydrolytic activities towards Suc–$(Ala)_3$–*p*NA (○) and Gl–Phe–*p*NA (▲).

hydrolyzed Gl–Phe–*p*NA but at a lower rate. It showed no hydrolytic activity towards Bz–Arg–*p*NA, Leu–*p*NA, or z-Gly–Leu.

Table II shows the glycogen-increasing effects of the fractions. The effect of STA-protease at 10 μg/ml corresponded to that of insulin at 10 mU/ml or more and was about 1.9 times that

Table I. Enzymatic Activities of Fractions obtained from Pronase

	Specific activity (units/mg)				
	Suc-$(Ala)_3$-*p*NA	Gl-Phe-*p*NA	Bz-Arg-*p*NA	Leu-*p*NA	z-Gly-Leu
Pronase	0.03	0.02	0.89	10.04	2.33
AP-1	0.28	0.14	0.02	31.88	6.64
AP-2	0.32	0.14	0.01	28.17	3.83
SBTI-1	0.52	0.14	0.01	0.01	0.02
STA-protease	0.44	0.02	0	0	0

Table II. Glycogen-increasing Effects of Pronase, AP-1, SBTI-1, STA-protease, and Insulin

	Glycogen content (μg of glycogen/100 mg of wet diaphragm/45 min)	
	Treated	Control
Pronase	71± 9[a)]	51±3
AP-1	94± 8[a)]	60±2
SBTI-1	105± 6[a)]	60±2
STA-Protease	132±13[a)]	47±6
Insulin	120± 9[a)]	47±6

a) Significantly different from the control at $p<0.05$. Protease fractions and insulin were assayed at concentrations of 10 μg/ml and 10mU/ml, respectively. Results are the mean values±S.E. of four observations.

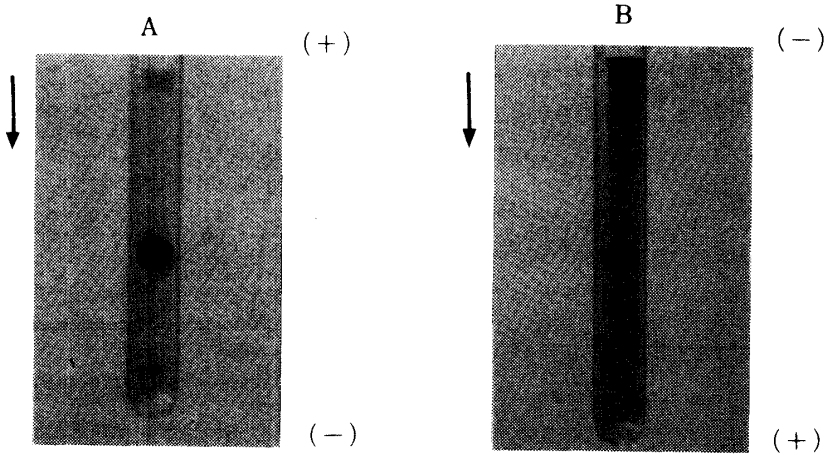

Fig. 3. Electrophoretic Patterns of STA-protease on Polyacrylamide Gel

A) Disc electrophoresis of STA-protease.
Sample: 100 μg of STA-protease.
Current: 4 mA per tube.
Migration: from anode to cathode.
B) SDS-polyacrylamide gel electrophoresis of guanidine-treated STA-protease
Sample: 40 μg of guanidine-treated STA-protease.
Current: 8 mA per tube.
Migration: from cathode to anode.

of Pronase. Two additional fractions, one adsorbed on AP–Sepharose and the other passing through SBTI–Sepharose, were assayed for glycogen-increasing activity at concentrations of 10 and 20 μg/ml. Little or no effect was found with these fractions.

Purity

STA-protease migrated as a single band in disc electrophoresis, and the guanidine-treated enzyme also gave a single band in electrophoresis on SDS-polyacrylamide gel (Fig. 3).

Molecular Weight

The molecular weight of STA-protease was estimated to be 20800 from a plot of the logarithm of the molecular weight *versus* the relative mobility with respect to protein standards in SDS-polyacrylamide gel electrophoresis (Fig. 4).

Stability and Kinetic Parameters

The effect of pH on the hydrolysis of Suc–$(Ala)_3$–*p*NA by STA-protease was investigated in 0.05 M Tris-HCl buffer adjusted from pH 2 to 12 with HCl or NaOH at 30°C for 30 min. The optimum pH was 8.5. No activity was found at pH 6.0 or less. The stability of the enzymatic activity to pH was determined after preincubation at 4°C for 1 h at various pH values. The enzyme was stable in the pH range from 2 to 10, whereas its activity was lost at pH 12. Figure 5 shows the stability of the enzyme to 8 M guanidine HCl at pH 2.5 and 8.1. On incubation at pH 2.5, the enzymatic activity decreased to 40% of the original level immediately after treatments at 0°C and 37°C and disappeared almost completely after 5 min at 37°C and 90 min at 0°C. At pH 8.1, the enzyme still possessed 47% of the original activity after treatment at 37°C for 90 min. In the case of non-treated enzyme, no change was found in its activity at either pH even after 90 min.

The values of K_m and K_{cat} for the hydrolysis of Suc–$(Ala)_3$–*p*NA and Ac–$(Ala)_3$–OMe were calculated from Lineweaver–Burk plots at substrate concentrations of 2 to 10 mM and the results are shown in Table III. For comparison, the values of Proteinases A, C, D, and E

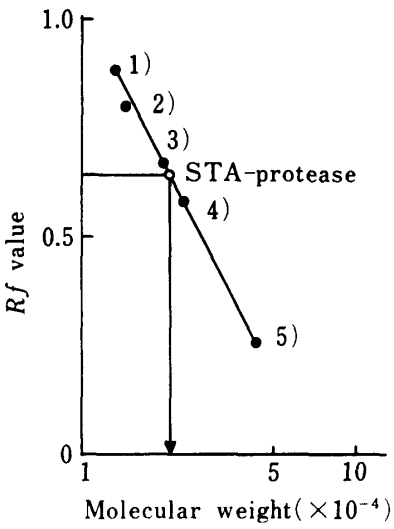

Fig. 4. Estimation of Molecular Weight of STA-protease by SDS-polyacrylamide Gel Electrophoresis

Electrophoresis was carried out as described in "Materials and Methods" and in the legend to Fig. 3.

Protein standards: 1) cytochrome C (molecular weight 13500), 2) lysozyme (14300), 3) SBTI (20100), 4) trypsin (23300), and 5) ovalbumin (43000).

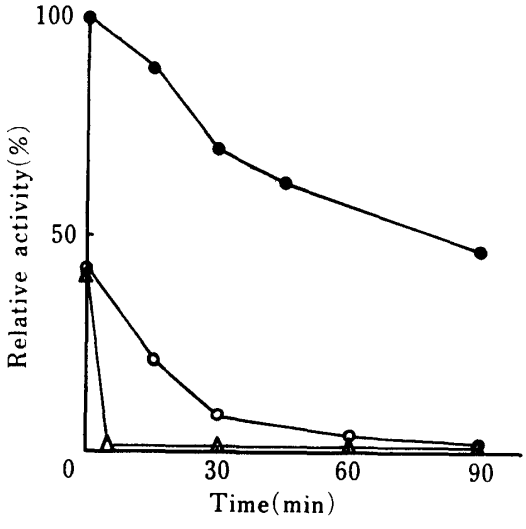

Fig. 5. Stability of STA-protease in the Presence of 8 M Guanidine HCl

STA-protease (0.38 mg/ml) was incubated in 5 mM Tris-HCl buffer, pH 8.1 containing 5 mM KCl, 10 mM $CaCl_2$, and 8 M guanidine HCl at 37°C (●). At pH 2.5, the enzyme (2.0 mg/ml) was incubated in 8 M guanidine HCl at 0°C (○) and 37°C (△). The hydrolytic activity towards Suc–$(Ala)_3$–*p*NA was determined as described in "Materials and Methods." The ordinate shows the relative activity with respect to the control which was incubated in the absence of guanidine HCl.

Table III. Kinetic Parameters of STA-protease and Proteinases A, C, D, and E

	Suc-(Ala)$_3$-*p*NA			Ac-(Ala)$_3$-OMe		
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m
STA-Protease	12	0.8	0.07	10.4	17.8	1.7
Proteinase A				13.3	101.6	7.6
Proteinase C				6.3	57.6	9.2
Proteinase D	3.3	0.3	0.1	0.8	1850	2313
Proteinase E	1.4	1.4	1.0	0.7	1400	2000

Suc-(Ala)$_3$-*p*NA and Ac-(Ala)$_3$-OMe (2-10 mM) were incubated with STA-protease in 0.1M Tris-HCl buffer at pH 8.3 and 25°C and at pH 8.0 and 30°C, respectively. Data for Proteinases A, C, D, and E are cited from Reference 26.

Table IV. Amino Acid Composition of STA-protease

Amino acid	Residues per mol[a]	Nearest integer
Lysine	1.0	1
Histidine	1.9	2
Arginine	7.9	8
Aspartic acid	17.5	18
Threonine[b]	31.2	31
Serine[b]	23.9	24
Glutamic acid	3.7	4
Proline	6.2	6
Glycine	35.8	36
Alanine	15.8	16
Half-cystine[c]	3.9	4
Valine	13.5	14
Methionine	1.9	2
Isoleucine	6.5	7
Leucine	6.9	7
Tyrosine	10.0	10
Phenylalanine	5.2	5
Tryptophan[d]	2.3	2
Total		197

a) Maximum value obtained after 12, 24, 48, and 72 h of hydrolysis, calculated on the basis of a molecular weight of 20800.
b) Values obtained by extrapolation to zero time of hydrolysis.
c) Determined as cysteic acid.
d) Determined by spectrophotometric analysis.

reported by Narahashi and Yoda[26] are also listed. The values of K_{cat}/K_m of Proteinases D and E for Ac-(Ala)$_3$-OMe were one thousand or more times greater than that of STA-protease, whereas the values of Proteinases A and C were not very different from that of STA-protease. The values of Proteinases D and E for Suc-(Ala)$_3$-*p*NA were higher than that of STA-protease.

Amino Acid Composition

Table IV shows the amino acid composition of STA-protease. The sum of amino acid residues was 92.6%. No carbohydrate was detected in the HCl-hydrolysate of STA-protease by thin layer chromatography.

Amino Acid Sequence

The amino acid sequences up to nine and thirty-two residues from the N-terminus were determined with guanidine-treated STA-protease and with *S*-carboxymethylated STA-protease, respectively (Table V). Repetitive yields were calculated to be 85—94%. An identical

Table V. Amino Acid Sequence from the N-Terminus of STA-protease

	Amino acid sequence
Guanidine-treated STA-protease	1 6 Ile-Ser-Gly-Gly-Asp-Ala-Ile-Tyr-Ser-
Yield (%)	52 —[a] 30 25 13 16 21 19 —
S-Carboxymethylated STA-protease	1 6 Ile-Ser-Gly-Gly-Asp-Ala-Ile-Tyr-Ser-Ser
Yield (%)	64 — 48 47 27 38 43 26 — —
	11 16 Thr-Gly-Arg-Cys-Ser-Leu-Gly-Phe-Asn-Val-
	— 17 6 4 — 15 15 18 — 17
	21 26 Arg-Ser-Gly-Ser-Thr-Tyr-Tyr-Phe-Leu-Thr-
	6 — 11 — — 8 13 9 9 —
	31 Ala-Gly-
	7 7

a) Detected by thin layer chromatography and not quantitated.

Table VI. Carboxypeptidase Digestion of STA-protease

Enzyme used for digestion	Incubation time (min)	nmol per nmol of substrate			
		Try	Phe	Val	Leu
DFP-treated Carboxy-peptidase A	10	0.51	0.23	0.15	0.13
	60	1.09	0.34	0.80	0.42
	180	2.03	0.61	1.42	0.86
Carboxy-peptidase C_{ua}	60	0.43	0.31	0.26	0.24
	180	1.37	0.69	0.98	0.66

sequence was obtained for the first nine residues of both modified enzymes. The N-terminal residue was identified as isoleucine.

The C-terminal amino acid residue was determined by digestion of guanidine-treated STA-protease with carboxypeptidase A and carboxypeptidase C_{ua} (Table VI). The results show that tyrosine (which is thought to be the C-terminus), phenylalanine, leucine, and valine residues exist in the C-terminal region.

Discussion

STA-protease was purified about 15-fold in terms of Suc–$(Ala)_3$–*p*NA– hydrolyzing activity with a protein yield of 6% from Pronase; in contrast, its glycogen-increasing activity showed only a 1.9-fold increase. This suggests that Pronase contains a protease(s) possessing insulin-like activity other than STA-protease. In fact, when SBTI-1 fraction was chromatographed on a CM-cellulose column by the method of Narahashi and Yoda,[26] two active fractions, one stable at pH 1.5 and the other unstable, were obtained (data not shown). Both fractions had the ability to hydrolyze Suc–$(Ala)_3$–*p*NA. The stable fraction showed a glycogen-increasing effect which was stronger than that of the unstable fraction. These results suggest that the insulin-like activity of Pronase is mainly attributed to the STA-ptorease which is stable at acidic pH. This explains why acid-treated Pronase still shows the insulin-like activity.[6]

Gertler and Trop[27] reported an early purification of three elastase-like enzymes, Enzymes I, II, and III, from Pronase B by chromatography on DEAE- and CM-cellulose. All three

Table VII. Similarities among Proteinases A, B, C, D, and E and Other Proteases

Proteinase	Other proteases	References
A	Enzyme A_1	29)
	Streptomyces griseus Protease A	28)
	Elastase-like Enzyme II	27)
	Lysine-free Chymoelastase	30)
B	*Streptomyces griseus* trypsin	28)
	Enzyme A_2	29)
C	Elastase-like Enzyme III	27)
	Protease B	28)
	Guanidine-stable Chymoelastase	30)
D	Elastase-like Enzyme I	27)
	Subtilisin-like enzyme	29)
E		

Results are summarized on the basis of the similarities pointed out by Narahashi and Yoda.[26]

enzymes hydrolyzed Ac–$(Ala)_3$–OMe, and the latter two also solublized congo red elastin. Jurášek *et al.*[28] studied a single-column preparative-scale fractionation of Pronase B on CM–Sephadex using a linear gradient of a volatile buffer system of pH 5.0 and found three major endopeptidases, *Streptomyces griseus* Proteases A and B and trypsin. The former two hydrolyzed acetyl-L-tyrosine ethylester (Ac–Tyr–OEt). Awad *et al.*[29] reported a partial purification of four serine endopeptidases which hydrolyzed Ac–Tyr–OEt or benzoyl-L-arginine ethylester (Bz–Arg–OEt) from Pronase B by chromatography on CM-cellulose and gel filtration on Sephadex G-75. The two enzymes which hydrolyzed Ac–Tyr–OEt were named lysine-free and guanidine-stable Chymoelastase.[30] Narahashi and Fukunaga[31] and Narahashi and Yoda[26] found that Pronase P contained at least five DFP-sensitive proteinases which were termed Proteinases A, B, C, D, and E. The enzymes were separated by chromatography on CM-Sephadex, CM-cellulose, and gel filtration on Sephadex, then lyophilized after dialysis. Narahashi and Yoda[26] pointed out the similarities among these and other proteases as summarized in Table VII. Proteinase E was thought to be a new type of protease not resembling the other enzymes. However, heterogeneity in molecular weight and amino acid composition often exists among enzymes which are classified into the same category. This is due presumably to autolytic digestion during fractionation and dialysis or to the presence of impurities in samples obtained by simplified methods. In the present study, an efficient affinity chromatography using 1 or 5 mM HCl as the eluent was used for the purification of STA-protease in order to overcome these disadvantages. Further, the purification procedures were devised to proceed without interruption and the final product was lyophilized without dialysis. There should, therefore, have been no chance of autolytic digestion, and STA-protease obtained was indeed clearly homogeneous in gel electrophoresis. The similarity of the values of K_{cat}/K_m shown in Table III suggests that STA-protease resembles Proteinase A or C. Proteinases D and E were unstable at acidic pH, unlike STA-protease.[26] The activity profile of STA-protease in the presence of 8 M guanidine HCl at pH 8.1 was quite similar to that of guanidine-stable Chymoelastase.[32] One lysine and four half-cystine residues were detected in both STA-protease and Protease B.[33] The minimum molecular weight of STA-protease was calculated to be 21012 from the content of lysine. This value was in good agreement with the value of 20800 obtained by SDS-polyacrylamide gel electrophoresis. This is slightly higher than the value of 18412 calculated for Protease B by Jurášek *et al.*,[34] who also determined its complete amino acid sequence. The N-terminal sequence up to residue 32 of STA-protease determined in the present study was identical with that of Protease B. The C-

terminal residue was tyrosine for both enzymes. However, phenylalanine and leucine residues which were found upon carboxypeptidase digestion of STA-protease did not exists in the sequence of eight residues from the C-terminus, –Leu–Ser–Val–Tyr–Gly–Ala–Ser–Val–Tyr, of Protease B. Moreover, serine, alanine, and glycine residues existing in the C-terminal region of Protease B were not detected in that of STA-protease. This is presumably due to the difference in the quality of the starting materials used for purification, that is, Pronase E for STA-protease and Pronase B for Protease B.[34]

In conclusion, the insulin-like activity of Pronase may be mainly attributed to STA-protease, and this enzyme is essentially homologous with Protease B, though there may be differences in the C-terminal region.

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