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Insulin-like Activity of Proteases. II.[1] A Protease possessing Insulin-like Activity in Pronase[2]

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The insulin-like activity of proteases was assayed in terms of the glycogen-increasing effect on hemidiaphragms isolated from mice. This effect was observed with Pronase, subtilisin BPN′, Sfericase, Dispase II, trypsin, α-chymotrypsin, and elastase, but not with pepsin, kallikrein, or lysozyme. Pronase, a mixed protease preparation from *Streptomyces griseus*, lost its insulin-like activity on treatment with diisopropyl fluorophosphate (DFP), whereas no loss of activity was observed with 1-chloro-3-tosylamide-7-amino-2-heptanone (TLCK) or L-(1-tosylamide-2-phenyl)-ethyl chloromethyl ketone (TPCK). An insulin-like activity-possessing protease (ILAPP) was partially purified from TLCK and TPCK-pretreated Pronase by affinity chromatography on soybean trypsin inhibitor-conjugated carriers. This enzyme migrated as a single band with a faint sub-band in disc electrophoresis, though it sedimented as a single peak on ultracentrifugal analysis. It hydrolyzed succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide and acetyl-L-alanyl-L-alanyl-L-alanine methyl ester at relatively high rates at pH 9, whereas the hydrolytic activities towards casein and elastin-Congo Red were low. It was strongly inhibited by DFP and phenylmethane sulfonyl fluoride (PMSF) but not by ethylenediaminetetraacetic acid, *p*-chloromercuribenzoic acid, monoiodoacetic acid, N-ethylmaleimide, or dithiothreitol. $HgCl_2$ was inhibitory at a concentration of 10^{-3} M. It is suggested that ILAPP is a DFP- and PMSF-sensitive alkaline protease and that the proteolytic activity of ILAPP is responsible for the insulinlike activity of Pronase.

Keywords——protease; insulin-like activity-possessing protease; diisopropyl fluorophosphate-sensitive protease; Pronase; glycogen-increasing effect; mouse hemidiaphragm

Insulin-like effects of several proteases have been reported, including the stimulation of glucose uptake, glycogen synthesis, antilipolysis, and incorporation of amino acids into protein with the use of isolated diaphragms,[4a–c] fat cells,[4c,5a–d] and isolated frog sartorius muscles.[6] We have also reported in a previous paper[1] that Pronase, a mixed protease preparation from *Streptomyces griseus*, showed an insulin-like effect on glucose uptake by hemidiaphragms isolated from mice. It has been considered in general that these effects are partly due to a limited modification of the cell surface caused by proteolytic processes.[5b,c] However, acid-treated Pronase which had lost more than 95% of its original proteolytic activity has been shown to retain the insulin-like activity of the native enzyme.[1,5a] It has thus been unclear whether or not the insulin-like activity of Pronase was produced by a mechanism similar to that of other proteases. The purpose of the present work was to

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2) This work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1977.
3) Location: *5-1, Oe-Honcho, Kumamoto 862, Japan.*
4) *a*) P. Rieser and C.H. Rieser, *Proc. Soc. Exptl. Biol. Med.*, **16**, 669 (1964); *b*) P. Rieser, *Acta Endocrinologica*, **54**, 375 (1967); *c*) T. Sakai, V.R. Lavis, J.W. Ensinck, and R.H. Williams, *Proc. Soc. Exptl. Biol. Med.*, **145**, 1096 (1974).
5) *a*) J.F. Kuo, C.E. Holmlund, I.K. Dill, and N. Bohonos, *Arch. Biochem. Biophys.*, **117**, 269 (1966); *b*) J.F. Kuo, I.K. Dill, and C.E. Holmlund, *J. Biol. Chem.*, **242**, 3659 (1967); *c*) T. Kono and F.W. Barham, *ibid.*, **246**, 6204 (1971); *d*) P. Cuatrecasas, *ibid.*, **246**, 6522 (1971).
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clarify this problem by the partial purification of a protease possessing insulin-like activity in Pronase.

Experimental

Chemicals——Pronase (*Streptomyces griseus* protease, Type E, 70 PUK/mg) was kindly provided by Dr. T. Kato of Kaken Chemical. Kallikrein (from hog pancreas, 1111 KU/mg) was donated by Professor C. Moriwaki of the Science University of Tokyo. Dispase II (*Bacillus polymyxa* protease, 300 PU/mg) was kindly provided by Dr. T. Irie of Godo Shusei. Elastase (from hog pancreas, 340 EL.U/mg), α-chymotrypsin (from bovine pancreas, 6.7 CHU/mg), and lysozyme (from egg white, 50 U/mg) were gifts from Eisai. Sfericase (*Bacillus spharicus* protease, 5000 U/mg) was a gift from Meiji Seika. Subtilisin BPN′ (*Bacillus subtilis* alkaline protease, 500 PUK/mg) was obtained from Nagase Sangyo. Trypsin (from bovine pancreas, Type I, 9700 BAEE U/mg), pepsin (from hog stomach mucosa, 4108 U/mg), insulin (from bovine pancreas, 24 IU/mg), soybean trypsin inhibitor (SBTI), and diisopropyl fluorophosphate (DFP) were obtained from Sigma Chemical. Phenylmethane sulfonyl fluoride (PMSF) was a product of Eastman Kodak. 1-Chloro-3-tosylamide-7-amino-2-heptanone (TLCK) and L-(1-tosylamide-2-phenyl)-ethyl chloromethyl ketone (TPCK) were purchased from Nakarai Chemical. Sephadex G-200 and Sepharose 4B were obtained from Pharmacia Fine Chemicals. Substrates used were as follows: succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Suc-$(Ala)_3$-*p*NA), benzoyl-DL-arginine *p*-nitroanilide (Bz-Arg-*p*NA), and benzoyl-L-tyrosine *p*-nitroanilide (Bz-Tyr-*p*NA) from the Protein Research Foundation; glutaryl-L-phenylalanine *p*-nitroanilide (Gl-Phe-*p*NA) from E. Merck; acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Ac-$(Ala)_3$-OMe) from Sigma Chemical; elastin-Congo Red from Seikagaku Kogyo; casein from Wako Pure Chemical. All other chemicals used were of analytical grade.

Animals——Mice, dd males weighing about 20 g, were fed a laboratory chow (MF, Oriental Yeast) for at least three days and fasted for 18 to 20 hr before use.

Preparation of SBTI-conjugated Carriers——Sephadex G-200 and Sepharose 4B, washed repeatedly with 0.1 M NaCl, were activated with cyanogen bromide at pH 11 according to the method of Cuatrecasas *et al.*[7] and coupled to SBTI in 0.1 M $NaHCO_3$, pH 8.5, by the method of Feinstein.[8]

Disc Electrophoresis——Electrophoresis on polyacrylamide gel was carried out in 0.13 M acetate buffer, pH 4.5, at a constant current of 4 mA per tube for 3 hr, essentially by the method of Reisfeld *et al.*[9] The concentration of the running gel was 7%. The gel was stained with 1% Amidoschwarz 10B and destained with 7% acetic acid.

Ultracentrifugation——Ultracentrifugation was carried out in a Hitachi UCA-1A ultracentrifuge. Insulin-like activity-possessing protease (ILAPP) was dissolved in 0.2 M phosphate buffer, pH 7.6, and dialyzed against the same buffer overnight. The final concentration of protein was 0.32%. Sedimentation measurements were made at a rotor speed of 60000 rpm.

Bioassay——The insulin-like activity was assayed in terms of the glycogen-increasing effect on hemidiaphragms isolated from mice. The assay method[1] reported previously was modified. Two hemidiaphragms were isolated from mice and incubated at 37° in Krebs-Ringer bicarbonate buffer, pH 7.9, containing a desired concentration of glucose, under an atmosphere of 95% O_2–5% CO_2 with shaking at 120 strokes/min for 90 min. After incubation, the glycogen content of the two hemidiaphragms was determined by the method of Seifter *et al.*[10]

Determination of Enzymatic Activities——Proteolytic activity was determined with 1.7% casein in 0.1 M Tris-HCl buffer containing 0.1 M NaCl, pH 8 or 10.[11] The hydrolytic activity towards elastin-Congo Red was determined at a concentration of 0.1% in 0.14 M borate buffer, pH 8.7.[12] Hydrolytic activities towards 0.2 mM Bz-Arg-*p*NA and Bz-Tyr-*p*NA[13] and 1 mM Gl-Phe-*p*NA[14] were determined in 0.05 M Tris-HCl buffer containing 5 mM $CaCl_2$, pH 8. Dimethyl sulfoxide was used, if necessary, to dissolve the substrate. Incubation was carried out for 30 min at 37°. The rate of hydrolysis of Suc-$(Ala)_3$-*p*NA was determined at a concentration of 1 mM in 0.05 M Tris-HCl buffer, pH 9.[15] An aliquot of 0.04 ml of 125 mM Suc-$(Ala)_3$-*p*NA dissolved in N-methyl-2-pyrrolidone and enzyme solution (0.2 ml in general) were added to 4.8 ml of the Tris-HCl buffer and the mixture was incubated for 30 min at 30°. After adding 0.2 ml of acetic acid to the

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incubated mixture, the absorbance was measured as rapidly as possible at 410 nm in a Hitachi 101 spectrophotometer. Esterolytic activity was measured by a colorimetric method[16] with 5 mM Ac-$(Ala)_3$-OMe as a substrate. Incubation was carried out in 0.1 M Tris-HCl buffer containing 0.1 M NaCl, pH 9, for 30 min at 30°.

Preparation of Pronase treated with TLCK, TPCK, or DFP——Pronase was treated with TLCK,[17] TPCK,[18] or DFP,[19] by slight modifications of the original methods. TLCK (2.28 mg, fine powder) or TPCK (1.12 mg in 0.028 ml of methanol) was added to 10 mg of Pronase dissolved in 1.8 ml of 0.067 M phosphate buffer, pH 7.9. The mixture was incubated for 2 hr with shaking at 37° and used as a sample for assay. For the preparation of DFP-Pronase, the amount of DFP required to inhibit Pronase was investigated prior to the preparation. As a result, 4.6 mg of DFP (32 mg/ml in isopropanol) was added to 5 ml of Pronase solution (10 mg/ml in 0.067 M phosphate buffer, pH 7.9). The mixture was incubated for 2 hr with shaking at 4°, dialyzed against deionized water for 48 hr, then lyophilized. Non-treated Pronase which had been incubated for 2 hr with shaking at 4° without the modifiers was used as a control.

Reaction with Chemical Reagents——A stock solution of DFP or PMSF dissolved in isopropanol was adjusted to concentrations of 2×10^{-2}, 2×10^{-3}, and 2×10^{-4} M. An aliquot of 0.01 ml of one of these solutions was added to 0.2 ml of ILAPP solution (50 µg/ml in 0.05 M Tris-HCl buffer, pH 9), and the mixture was preincubated for 2 hr with shaking at 30°. After dilution with 4.8 ml of the same buffer, an aliquot of 0.04 ml of 125 mM Suc-$(Ala)_3$-*p*NA was added to determine the enzymatic activity, as described previously. $HgCl_2$, ethylenediaminetetraacetic acid (EDTA), *p*-chloromercuribenzoic acid (PCMB), monoiodoacetic acid (MIA), N-ethylmaleimide (NEM), and dithiothreitol (DTT) were dissolved separately in 0.05 M Tris-HCl buffer, pH 9, at concentrations of 2×10^{-3}, 2×10^{-4}, and 2×10^{-5} M. An equal volume of ILAPP solution (4 µg/ml in the same buffer) was added to 2.5 ml of each solution, and the mixture was preincubated for 1 hr with shaking at 30°. After addition of 0.04 ml of 125 mM Suc-$(Ala)_3$-*p*NA, the enzymatic activity was determined as described previously. For each control, ILAPP was preincubated under the same conditions, except for omission of the reagents.

Results and Discussion

Effects of Insulin and Various Enzymes on the Glycogen Content of Hemidiaphragms

The effect of insulin on the glycogen content of hemidiaphragms isolated from mice was investigated in the concentration range of 0.1 to 1000 mU/ml. As shown in Fig. 1, the net increase in glycogen (Treated–Control) was proportional to the logarithmic concentration of insulin within the range of 1 to 1000 mU/ml.

Table I. Insulin-like Activities of Various Enzymes

	Net increase in glycogen[a]		
	1	10	50(µg/ml)[b]
Pronase	6±7(3)[c]	83±9(3)	20±10(3)
Dispase II	16±5(4)	24±7(4)	162±12(4)
Sfericase	19±5(3)	42±2(3)	−13± 8(3)
Subtilisin BPN′	5±5(4)	24±6(4)	34± 6(4)
Trypsin	10±9(4)	70±7(4)	59± 7(4)
α-Chymotrypsin	24±5(3)	40±8(7)	59±11(3)
Elastase	−9±7(4)	44±6(4)	112±15(4)
Pepsin	−5±5(3)	4±9(9)	−7±11(5)
Kallikrein	−7±7(3)	−11±5(3)	−10± 5(3)
Lysozyme	−6±6(3)	−7±8(3)	−6± 8(3)

a) µg of glycogen per 100 mg of wet hemidiaphragms per 90 min; assayed at a glucose concentration of 33 mM and calculated as described in the legend to Fig. 1.
b) Concentration of enzyme.
c) The mean value ± S.E. (the number of observations).

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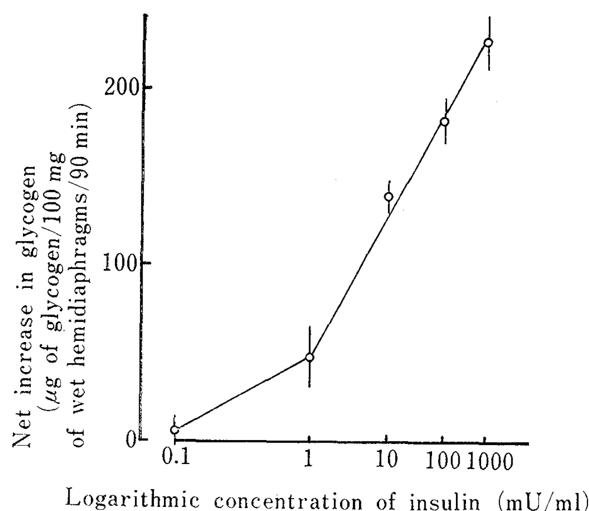

Fig. 1. Effect of Insulin on the Glycogen Content of Hemidiaphragms

Insulin and two hemidiaphragms were incubated with 33 mM glucose as described in the text. The effect of insulin is shown in terms of the net increase in glycogen (μg of glycogen per 100 mg of wet hemidiaphragms per 90 min) obtained by subtracting the glucose content of the control from that of the treated material. Each point represents the mean value ± S.E. of three to five observations.

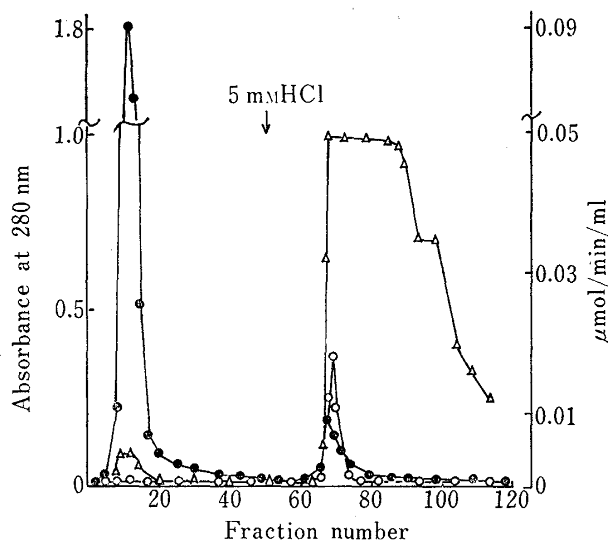

Fig. 2. Affinity Chromatography of Pronase on SBTI-Sepharose 4B

Pronase (70 mg) was applied to the column and eluted with 1/15 M phosphate buffer, pH 7.9, and then with 5 mM HCl at a flow rate of 150 ml/hr. The effluent was collected in 5 ml fractions. When the column was eluted with 5 mM HCl, fractions were collected in tubes containing 2 ml of the phosphate buffer. ●, absorbance at 280 nm; ○, hydrolytic activity towards Gl-Phe-*p*NA; △, hydrolytic activity towards Bz-Arg-*p*NA.

Table I shows the net increase in glycogen when various enzymes were incubated with the hemidiaphragms at concentrations of 1, 10, and 50 μg/ml. A glycogen-increasing effect was observed with Pronase, Sfericase, Dispase II, subtilisin BPN′, trypsin, elastase, and α-chymotrypsin, but not with pepsin, kallikrein, or lysozyme. The values for Pronase, Sfericase, and trypsin were lower at 50 μg/ml than at 10 μg/ml, probably due to destruction of the tissue by their proteolytic action. Although Rieser[4b] reported that pepsin and pepsinogen stimulated the uptake of 3-O-methyl glucose and promoted the synthesis of glycogen from glucose on diaphragms isolated from rats, we were unable to detect glycogen-increasing effects of pepsin on hemidiaphragms isolated from mice. Frey *et al.*[20] reported that kallikrein was effective at a dose of 0.005 KU/kg in the *in vivo* assay using arterial blood pressure of animals and at a level of 0.002 KU in the *in vitro* assay on isolated guinea pig ileum. In the present study, therefore, further tests on the glycogen-increasing effect were carried out at much lower concentrations of kallikrein (0.005—0.1 μg/ml, corresponding to 0.005—0.1 KU). However, no effect was observed at any concentration tested. These results suggest that the concentration appropriate for the bioassay of protease may be within the range of 10 to 50 μg/ml in the incubation medium.

Affinity Chromatography of Pronase on SBTI-Sepharose 4B

Pronase (70 mg) was applied to a 2×17.5 cm column of SBTI-Sepharose 4B and eluted with 0.067 M phosphate buffer, pH 7.9, and then with 5 mM HCl (Fig. 2). Two peaks, the first passing through the column and the second being eluted with 5 mM HCl, were obtained in terms of absorbance at 280 nm. The hydrolytic activity towards Gl–Phe–*p*NA was concentrated into the second peak, though the profile of the activity did not exactly parallel that of protein. The activity towards Bz–Arg–*p*NA, on the other hand, appeared with the second peak and was also detected in fractions far behind the peak. No or little activity towards

20) E.K. Frey, H. Kraut, and E. Werle, "Das Kallikrein-Kinin-System und seine Inhibitoren," Ferdinand Enke Verlag, Stuttgart, 1968, pp. 10—17.

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either substrate was observed in the first peak. These results suggest that proteases possessing high hydrolytic activity towards Bz–Arg–*p*NA or Gl–Phe–*p*NA may bind to SBTI more tightly than the proteases contained in the second peak. Fractions P–I (fraction numbers 8—14), P–II (67—73), and P–III (89—103) were collected and used as samples for the determination of insulin-like end enzymatic activities. Yields of P–I, P–II, and P–III were 63, 8, and 1%, respectively, based on the absorbances at 280 nm.

Table II. Insulin-like and Enzymatic Activities of Fractions obtained by Affinity Chromatography of Pronase on SBTI-Sepharose 4B

	Glycogen content[a]		Enzymatic activity		
	Treated	Control	Casein	Bz–Arg–*p*NA	Gl–Phe–*p*NA
P-I	150± 9 (6)[b]	146± 9(6)	1.0[c]	3[d]	0.4[d]
P-II	314±10*(5)	153± 9(5)	2.6	400	56
P-III	174±10 (6)	152±13(6)	2.0	4100	5

a) μg of glycogen per 100 mg of wet hemidiaphragms per 90 min; determined at a glucose concentration of 33 mM in the presence (Treated) or absence (Control) of 10 μg/ml of each fraction.
b) The mean value ± S.E. (the number of observations)
c) μmol of tyrosine relased per min per mg.
d) nmol of *p*-nitroaniline released per min per mg. *$p < 0.01$, as compared to the control.

Of the three fractions, P–II showed the most potent increasing effect on the glycogen content of hemidiaphragms (Table II). The effect was statistically significant as compared to the control. Although the hydrolytic activity of P–III towards Bz–Arg–*p*NA was extremely high, its glycogen-increasing effect was much lower than that of P–II. No glycogen-increasing effect was observed with P–I.

Table III. Insulin-like and Enzymatic Activities of the Pronase treated with TLCK, TPCK, or DFP

	Glycogen content[a]		Enzymatic activity			
	Treated	Control	Casein	Bz–Arg–*p*NA	Gl–Phe–*p*NA	Suc–$(Ala)_3$–*p*NA
Non-treated Pronase	271±25*(5)[b]	175±15(5)	2.9[c]	338[d]	16[d]	95[d]
TLCK-Pronase	250±16*(6)	156± 8(6)	1.5	1	12	100
TPCK-Pronase	304±25*(5)	167±11(5)	0.7	192	9	49
DFP-Pronase	170±16 (5)	152±14(5)	0.6	14	1	4

TLCK-, TPCK-, and DFP-Pronase denote the Pronase preparations treated with TLCK, TPCK, and DFP, respectively.
a) μg of glycogen per 100 mg of wet hemidiaphragms per 90 min; determined at a glucose concentration of 33 mM in the presence (Treated) or absence (Control) of 10 μg/ml of enzyme.
b) The mean value±S.E. (the number of observations).
c) μmol of tyrosine released per min per mg.
d) nmol of *p*-nitroaniline released per min per mg.
* $p < 0.01$, as compared to the control.

Insulin-like and Enzymatic Activities of Pronase treated with TLCK, TPCK, or DFP

Table III compares the insulin-like and enzymatic activities of the TLCK-, TPCK-, and DFP-treated Pronase preparations. On treatment with TLCK, the hydrolytic activities towards Bz–Arg–*p*NA and casein were decreased to 0.3 and 52% of the original, respectively. TPCK produced a decrease in hydrolytic activities towards all four substrates used. Both TLCK- and TPCK-Pronase, however, retained the original insulin-like activity. No

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insulin-like activity was observed with DFP-Pronase, which showed much lower activity towards all the substrates used than TLCK- and TPCK-Pronase. These results suggest that the protease possessing insulin-like activity in Pronase may be a kind of DFP-sensitive protease (s) which is different from either the TLCK- or the TPCK-sensitive protease.

An Insulin-like Activity-possessing Protease

Partial Purification——Pronase was treated with TLCK and TPCK prior to purification. Pronase (100 mg) in 18 ml of 0.067 M phosphate buffer, pH 7.2, was treated with 22.8 mg of TLCK and 11.2 mg of TPCK dissolved in 0.28 ml of methanol, and the mixture was incubated for 2 hr with shaking at 37°. The following procedure was carried out in order to remove active proteases sensitive to TLCK and TPCK remaining after the inactivation. Ten ml of SBTI-Sephadex G-200 equilibrated with 0.067 M phosphate buffer, pH 7.9, was added to the pretreated Pronase solution, stirred for 15 min in an ice-cooled bath, and filtered through a sintered-glass filter. The filtrate was used for the next purification step. The fraction adsorbed on SBTI-Sephadex G-200 was eluted with 5 mM HCl. The amount of protein in the eluate was 0.27 mg (yield, 0.4%). This fraction contained the active proteases, and its hydrolytic activities towards Gl–Phe–*p*NA, Bz–Arg–*p*NA, and Suc-$(Ala)_3$-*p*NA were 2.2, 13, and 93 (nmol/min/mg), respectively. This fraction was so small in amount that it could not be assayed for insulin-like activity.

The filtrate (68 ml) was applied to a 2×20.5 cm column of SBTI-Sepharose 4B equilibrated with 0.067 M phosphate buffer, pH 7.9 (Fig. 3). The column was washed with 200 ml of the same buffer and eluted with 10 ml HCl containing 0.2 M KCl. The profile of activity towards Suc–$(Ala)_3$-*p*NA paralleled that of protein. From 100 mg of Pronase, 6.9 mg of the final product was obtained. This preparation was called insulin-like activity-possessing protease (ILAPP). No insulin-like activity was found with the fraction which had passed through the column.

Purity——ILAPP migrated as a single band with a faint sub-band in disc electrophoresis, though it sedimented as a single peak in ultracentrifugal analysis (Fig. 4 and 5).

Insulin-like Activity——Table IV shows the glycogen-increasing effects of Pronase, ILAPP, and insulin on the hemidiaphragms. The hemidiaphragms were incubated at a glucose concentration of 5 mM in the presence or absence of enzyme and insulin. ILAPP

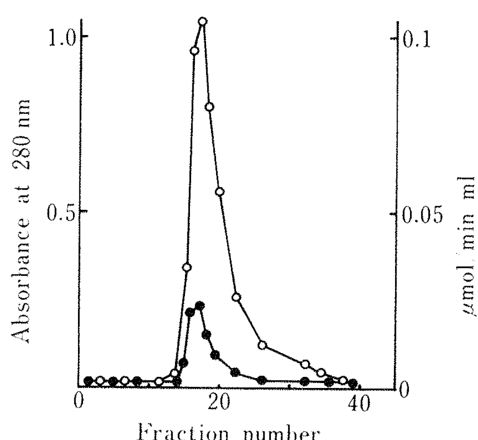

Fig. 3. Elution Profile of ILAPP from SBTI-Sepharose 4B

ILAPP was eluted from the column with 10 mM HCl containing 0.2 M KCl at a flow rate of 150 ml/hr. Fractions (5 ml) were collected in tubes containing 2 ml of 1/15 M phosphate buffer, pH 7.9.
●, absorbance at 280 nm; ○, hydrolytic activity towards Suc-$(Ala)_3$-*p*NA.

Fig. 4. Disc Electrophoresis of ILAPP

Migration: from top to bottom (cathode to anode). Sample: 200 μg of ILAPP.

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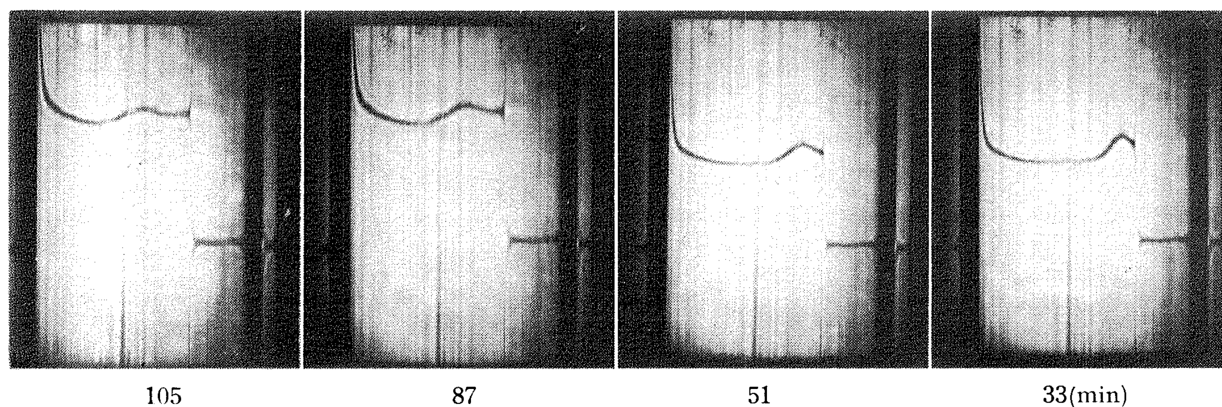

Fig. 5. Sedimentation Patterns of ILAPP

Sedimentation: from right to left.
Concentration of ILAPP: 0.32% in 0.2 M phosphate buffer, pH 7.6.
Rotor speed: 60000 rpm.

TABLE IV. Glycogen-increasing Effects of ILAPP, Pronase, and Insulin

	Concentration (μg/ml)	Glycogen content[a) Treated]	Glycogen content[a)] Control
ILAPP	2	91±11*(6)[b)]	52±5(6)
	10	136±18*(5)	56±4
Pronase	2	70± 9 (4)	48±7(4)
	10	105± 6*(4)	57±7(4)
Insulin	1[c)]	114±16*(5)	51±7(5)
	10	176±19*(4)	53±7(4)

a) μg of glycogen per 100 mg of wet hemidiaphragms per 90 min; determined at a glucose concentration of 5 mM in the presence (Treated) or absence (Control) of enzyme and insulin.
b) The mean value±S.E. (the number of observations).
c) mU/ml.
* $p < 0.01$, as compared to the control.

showed a more potent increasing effect than Pronase at a concentration of 10 μg/ml, though its effect was lower than that of 10 mU/ml of insulin.

Substrate Specificity——Table V compares the hydrolytic activities of ILAPP and other proteases towards various substrates. ILAPP hydrolyzed the substrates tested at appreciable rates, except for Bz–Arg–pNA and Bz–Tyr–pNA, which were hardly susceptible to hydrolysis by the enzyme. Suc–(Ala)$_3$-pNA and Ac–(Ala)$_3$-OMe were hydrolyzed at much higher rates than other substrates. This tendency was also seen with substilisin BPN' and elastase. However, the activities of ILAPP towards casein and elastin-Congo Red, which are good substrates for subtilisin BPN' and elastase, respectively, were quite low.

Effect of pH on Enzymatic Activity——The effect of pH on the enzymatic activity was determined with Suc–(Ala)$_3$-pNA and casein. The activity of ILAPP towards Suc–(Ala)$_3$-pNA was determined in 0.05 M Tris–HCl buffer, pH 8 and 9, and in 0.05 M bicarbonate buffer, pH 10 and 11. The activity towards casein was determined in 0.1 M Tris-HCl buffer containing 0.1 M NaCl adjusted to pH 8, 9, 10, 11, or 12 with 0.5 N NaOH. ILAPP showed the maximum activities at pH 9 towards Suc–(Ala)$_3$-pNA and pH 10 towards casein. Activities towards both substrates decreased rapidly on either side of the optimal pH.

Stability at Various pH Values——ILAPP was dissolved in 0.1 M Tris-HCl buffer containing 0.1 M NaCl, adjusted to pH 1.8, 4.6, 7.4, 9.2, 10.8, or 12.5 with 0.2 N HCl or 0.5 N NaOH, and preincubated for 1 hr at 4°. The enzymatic activity was determined in 0.1 M Tris-HCl buffer, pH 10, with casein as a substrate. ILAPP was stable at pH 7.4 and 9.2 and retained

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Table V. Substrate Specificities of ILAPP and Other Enzymes

	Enzymatic activity					
	ILAPP	Pronase	Subtilisin BPN′	Elastase	Trypsin	Chymotrypsin
Casein[a] (pH 8)	0.5	2.9	3.4	2.7	3.1	4.5
(pH 10)	0.6	0.6	2.6	0.7	1.2	3.5
Elastin-Congo Red[b]	0.8	1.1	2.6	38	0.1	0.1
Bz–Arg–*p*NA[c]	1.5	421	0	0	273	3.0
Bz–Tyr–*p*NA[c]	0	2.0	0	3.5	14	238
Gl–Phe–*p*NA[c]	4.1	21	2.9	5.7	2.0	29
Suc–$(Ala)_3$-*p*NA[c]	270	153	85	4274	0.3	1.7
Ac–$(Ala)_3$-OMe[d]	8.8	35	67	11	0	0.8

a) μmol of tyrosine released per min per mg.
b) μmol of Congo Red released per min per mg.
c) nmol of *p*-nitroaniline released per min per mg.
d) μmol of methanol released per min per mg.

Table VI. Effects of Chemical Reagents on the Enzymatic Activity of ILAPP

	Relative activity (%)		
	10^{-5}	10^{-4}	10^{-3} (M)
EDTA	103	108	100
$HgCl_2$	100	86	61
PCMB	114	90	90
MIA	92	91	91
NEM	91	91	98
DTT	87	92	94
DFP	57	7	0
PMSF	55	3	0

Relative activities are expressed as percentages of the activity of the control, which was determined after preincubation without the chemical reagent.

50 and 60% of the original activity after preincubation at pH 1.8 and 4.6, respectively. The remaining activity was only 10% at pH 10.8 and 12.5.

Effects of Chemical Reagents on the Enzymatic Activity——Table VI shows the effects of chemical reagents on the hydrolytic activity of ILAPP towards Suc–$(Ala)_3$-*p*NA. EDTA, PCMB, MIA, and NEM did not show distinct inhibition within the range of concentrations from 10^{-5} to 10^{-3} M. These results suggest that neither metal ions nor sulfhydryl groups are involved in the catalytic site of ILAPP. Inhibition was not observed with DTT, which would reduce a disulfide group to sulfhydryl groups. $HgCl_2$ was inhibitory at a concentration of 10^{-3} M, presumably by forming a stable chelate between active histidine and aspartic acid residues.[21] PMSF and DFP, which are known to react with active serine residues,[22] distinctly inhibited the activity of ILAPP. This suggests that a serine residue (s) may be involved in the catalytic site of ILAPP. ILAPP appeared to bind SBTI more weakly than trypsin- and chymotrypsin-like enzymes in Pronase, based on the results of affinity chromatography of native Pronase on SBTI-Sepharose 4B and the adsorption of TLCK- and TPCK-pretreated

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Pronase on SBTI-Sephadex G-200. It is not clear at present whether or not the binding of ILAPP occurs by a mechanism similar to that of trypsin. Narahashi and Yoda[23] reported that two DFP-sensitive alkaline proteases with strong esterase activity towards Ac-$(Ala)_3$-OMe, designated as alkaline serine proteinase D and E, were purified from Pronase P. Proteinase D seemed to be identical with a subtilisin-like enzyme demonstrated by Awad *et al.*[24] The substrate specificity of ILAPP resembles that of proteinase D or E. Further work, however, is required to identify these enzymes. Studies are now in progress to determine the chemical and physico-chemical properties of ILAPP for comparison with the properties of these enzymes. The observation that the acid-treated Pronase still showed the insulin-like activity of the native enzyme may be accounted for by the stability of ILAPP at acidic pH. The caseinolytic activity of ILAPP was much lower than that of Pronase. This may be one reason why the relationship between the insulin-like and proteolytic activities of Pronase is not yet established. The insulin-like activity of Pronase may be mainly due to ILAPP, which probably acts by causing a limited digestion of the cell surfaces.

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