Chem. Pharm. Bull.
28(6)1775—1781(1980)

Insulin-like Activity of Proteases. III.[1] Effect of Insulin-like Activity-possessing Protease on Vascular Permeability

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(Received December 19, 1979)

The insulin-like activity-possessing protease (ILAPP), which was partially purified from Pronase, showed a high but short-lived increasing effect on the vascular permeability of mice, whereas only a low hemorrhagic activity was detected at the same dose. Soybean trypsin inhibitor and leupeptin both inhibited this increasing effect, whereas they exerted no influence on the hydrolytic activity of ILAPP towards casein or succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide under the same conditions. The vasodilative activity assayed in a dog was very low, and no kinin-forming activity was detected. This enzyme showed a kinin-inactivating activity when preincubated with bradykinin. Its hydrolytic activity was higher towards acetyl-L-alanyl-L-alanyl-L-alanine methyl ester than towards derivatives of basic amino acid methyl esters. These results suggest that ILAPP differs from trypsin and trypsin-like enzymes in the specificity. The possibility that the insulin-like activity of ILAPP is attributable to kinins produced directly by ILAPP may therefore be excluded.

Keywords——protease; proteolytic activity; esterolytic activity; insulin-like activity; kinin-inactivating activity; vascular permeability-increasing activity

Insulin produces such diverse cellular effects as the stimulation of glucose and amino acid transport into cells, the promotion of glycogen and protein syntheses, and the inhibition of both lipolysis in fat cells and hepatic gluconeogenesis.[3] Moreover, it is considered that the initial step in these effects is probably the interaction of the hormone with specific receptors on the cell surface.[4] However, direct evidence for the mechanism of insulin action has not yet been obtained. Moriwaki *et al.*[5] reported first that glucose and amino acid transport across the rat small intestine was enhanced by kallikrein and bradykinin. Dietze *et al.*[6] reported recently that the glucose uptake stimulated by insulin was about halved during the infusion of trasylol, which is a kallikrein-trypsin inhibitor, when the arterial-deep venous concentration difference of glucose was measured in the human forearm. This result suggested that the kallikrein-kinin system is involved in the translation of insulin action on glucose metabolism in skeletal muscle. It was also reported, with a similar experimental model, that bradykinin itself increased glucose uptake into muscle in the same way as insulin.[7] We reported in a previous paper[1] that the insulin-like activity-possessing protease (ILAPP), which was partially purified from Pronase, showed a glycogen-increasing effect on hemidiaphragms isolated from mice and that its proteolytic action was responsible for the effect. There is, however, no direct evidence for the mechanism and no information on the production of kinins by ILAPP.

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The purpose of the present work was to clarify whether or not ILAPP itself produces kinins.

Experimental

Chemicals——Pronase (from *Streptomyces griseus*, type E, 70 PUK/mg) was kindly provided by Dr. T. Kato of Kaken Chemical. Alkaline protease inhibitor (API, from *Streptomyces pseudogriseolus*) was donated by Professor M. Shibata and Dr. M. Uyeda of this University. Habu toxin (from *Trimeresurus flavoviridis*) was a gift from the Chemo-Sero Therapeutic Research Institute. α-Chymotrypsin and elastase (from bovine pancreas) were gifts from Eisai. D-2-Bromolysergic acid diethylamide (BOL-148) was a gift from Sandoz Pharmaceuticals. Indomethacin was kindly provided by Nippon Merck-Banyu. Hydrocortisone was a gift from Nikken Kagaku. Subtilisin BPN′ (*Bacillus subtilis* alkaline protease) was obtained from Nagase Sangyo. Trypsin (from bovine pancreas) and soybean trypsin inhibitor (SBTI) were purchased from Miles Biochemicals. Pepstatin, leupeptin, synthetic bradykinin, succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Suc-$(Ala)_3$-pNA), tosyl-L-lysine methyl ester HCl (Tos-Lys-OMe), tosyl-L-arginine methyl ester HCl (Tos–Arg–OMe), acetyl-L-arginine methyl ester HCl (Ac–Arg–OMe), and acetyl-glycyl-L-lysine methyl ester (Ac–Gly–Lys–OMe) were purchased from the Protein Research Foundation. Acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Ac–$(Ala)_3$–OMe) and benzoyl-L-arginine methyl ester HCl (Bz–Arg–OMe) were obtained from Sigma Chemical. Casein, serotonin, and histamine were purchased from Wako Pure Chemical. Pontamine Sky Blue 6B was purchased from Tokyo Kasei Kogyo. All other chemicals used were of analytical grade.

Partial Purification of ILAPP——ILAPP was partially purified from Pronase pretreated with 1-chloro-3-tosylamido-7-amino-2-heptanone and L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone by affinity chromatography on SBTI-conjugated Sepharose 4B, as described in a previous paper.[1] The molecular weight of ILAPP was estimated to be roughly 21000 by electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate.[8]

Enzymatic Activity——Proteolytic activity was determined with 1.7% casein in 0.15 M Tris-HCl buffer containing 0.1 M NaCl, pH 7.4.[9] The hydrolytic activity towards Suc-$(Ala)_3$-pNA was determined at a concentration of 1 mM in 0.05 M Tris-HCl buffer, pH 9.[1] Esterolytic activity was determined by a colorimetric method[10] using Bz–Arg–, Tos–Arg–, Tos–Lys–, Ac–Arg–, Ac–Gly–Lys–, and Ac–$(Ala)_3$–OMe as substrates. Incubation was performed at 30° for 30 min in 0.05 M Tris-HCl buffer, pH 8, except that Ac–$(Ala)_3$–OMe was incubated in 0.1 M Tris-HCl buffer containing 0.1 M NaCl, pH 9. The activity is represented in terms of esterolytic units (EU) equal to μmoles of substrate hydrolyzed per min.

Vascular Permeability-increasing Activity——We used dd male mice weighing about 20 g, the abdomens of which were clipped and depilated with Eba depilatory cream one day previously, throughout this study. The mice each received an intravenous injection of 25 μl of a Pontamine Sky Blue 6B solution (50 mg/ml in 0.43% NaCl solution). Samples dissolved in a constant volume of 50 μl of physiological saline were injected intradermally into the abdomen. The animals were sacrificed 30 min after administration of the dye.[11] A standard area of skin was removed by means of a corkborer 14 mm in diameter and soaked in 1 ml of 1 N KOH at 37° for 16 hr according to the method of Katayama *et al.*[12] An aliquot of 9 ml of a mixed solution of 0.6 N phosphoric acid and acetone (5: 13, v/v) was added to this. After thorough mixing, the mixture was centrifuged at $1500 \times g$ for 15 min, and absorbance of the supernatant obtained was measured at 620 nm. The results are represented in terms of blueing activity (μg of dye leaked per site).

Hemorrhagic Activity——Hemorrhagic activity was measured by a simplification of the method reported by Kondo *et al.*[13] Samples dissolved in a constant volume of 50 μl of physiological saline were injected intradermally into the depilated abdomen of mice. The animals were sacrificed 14 hr later, and the skin was removed immediately. The skin was spread on a glass plate to retain its original size. The cross-diameters of hemorrhagic spots were measured from the reverse side of the skin. The results are represented in terms of the mean values of the cross-diameters, mm.

Vasodilative Activity——Vasodilative activity was assayed with adult dogs weighing 7—12 kg by the method of Moriwaki *et al.*,[14] using an electromagnetic flowmeter (MF-2, Nihon Kohden). The increase in blood flow was measured at the femoral artery of an anesthetized dog. ILAPP was dissolved in 0.1 ml of

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0.05 M phosphate buffer, pH 8, at a desired concentration and administered into the femoral artery. The activity is represented in terms of kallikrein units (KU).

Kinin-forming and Kinin-inactivating Activities——Kinin-forming activity was assayed in the following way.[14] Kininogen which had been prepared from heated bovine plasma was dissolved in 0.05 M phosphate buffer, pH 8, at a concentration of 45 mg/ml, and 0.3 ml of this solution was added to 0.1 or 0.2 ml of ILAPP. After incubation at 30° for 1 or 2 min, the incubated mixture was administered to a gut bath equipped with an ileum segment isolated from guinea pig, and the kinin produced was isometrically measured with a force-displacement transducer (SB-1T, Nihon Koden).

For measurement of the kinin-inactivating activity, ILAPP was incubated with 100 ng of bradykinin instead of the kininogen in the phosphate buffer at 30° for 2—4 min, and the residual bradykinin activity in the incubated mixture was assayed as described above. The activity is represented in terms of μg of bradykinin inactivated per min per mg.

Results

Vascular Permeability-increasing and Hemorrhagic Activities

The time course of blueing activity was investigated with serotonin and ILAPP (Fig. 1). Both substances showed maximum activity 30 min after injection into mice, and little activity was detected 60 and 240 min after injection of serotonin and ILAPP, respectively.

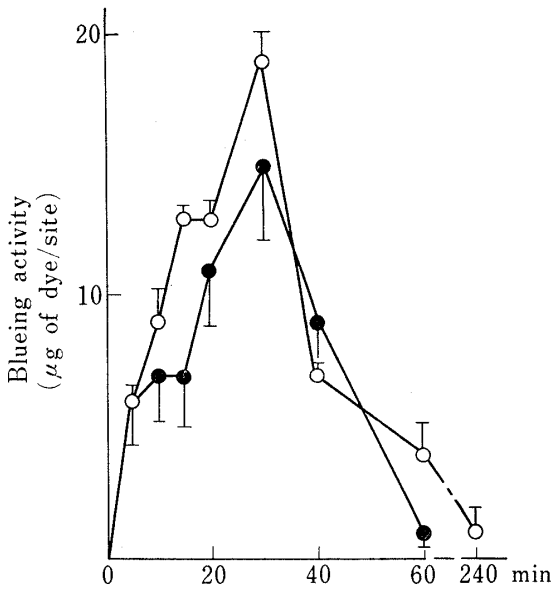

Fig. 1. Time Course of the Increasing Effects of ILAPP and Serotonin on the Vascular Permeability of Mice

○, ILAPP 0.5 mg/kg; ●, Serotonin 12.5 μg/kg. Blueing activity is represented in terms of the net amount of dye obtained by subtracting the control value from the treated value, obtained from the same mouse. Each point represents the mean value±S.E. of three observations.

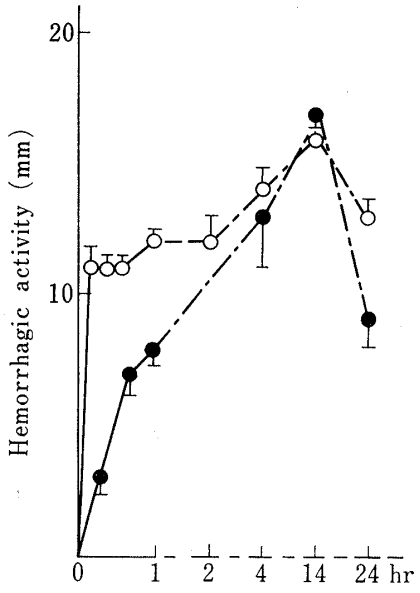

Fig. 2. Time Course of Hemorrhagic Activities of Pronase and Habu Toxin

○, Pronase 0.5 mg/kg; ●, Habu toxin 47.6 μg/kg. No hemorrhage was observed with the control, in which a constant volume of 50 μl of physiological saline was injected intradermally into the depilated abdomen. Each point represents the mean value±S.E. of three observations.

Figure 2 shows the time course of hemorrhagic activities of Pronase and Habu toxin. Both substances produced the most pontent hemorrhage 14 hr after injection, and their activities were lower at 24 hr. Thus, blueing and hemorrhagic activities were routinely measured at 30 min and 14 hr, respectively, after injection.

Dose-response courves for the blueing and hemorrhagic activities of ILAPP, serotonin, and Habu toxin are shown in Fig. 3. Increase in the blueing activity was proportional to dose in the ranges of 0.075 to 0.5 mg/kg for ILAPP, 1.5 to 12 μg/kg for serotonin, and 5 to 45

μg/kg for Habu toxin, whereas proportional increase was not observed with the hemorrhagic activities of ILAPP and serotonin, probably due to the very low activities. In the case of Habu toxin, the profile of the hemorrhagic activity paralleled that of the blueing activity, and an increase in the vascular permeability was accompanied by strong hemorrhage at the injection site. When the hemorrhagic activity was compared among various proteases at doses showing blueing activity in the range of 15 to 20 μg of dye/site, the activities (cross-diameters

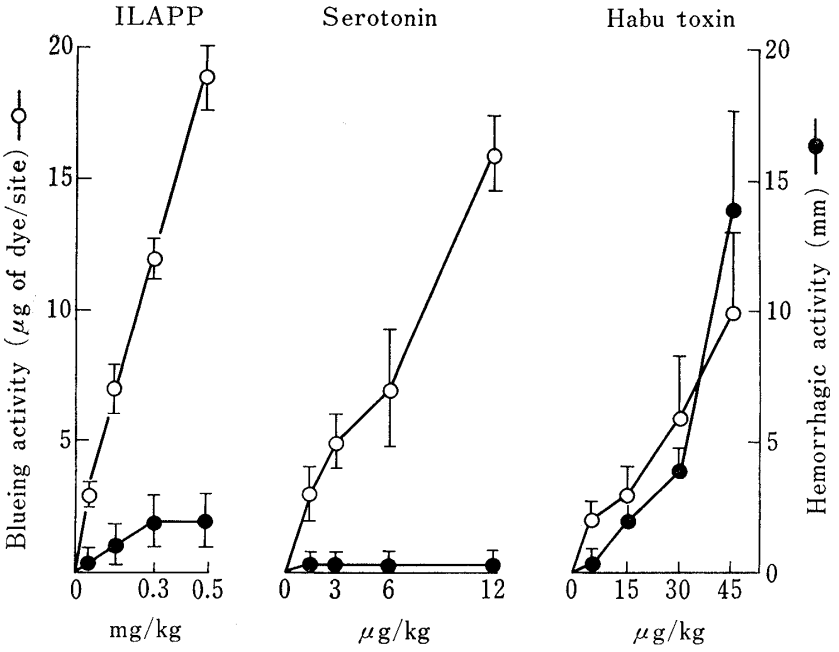

Fig. 3. Dose-response Courves for Vascular Permeability-increasing and Hemorrhagic Activities of ILAPP, Serotonin, and Habu Toxin

Blueing and hemorrhagic activities were assayed 30 min and 14 hr, respectively, after administration of samples. Each point represents the mean value±S.E. of three observations.

Table I. Inhibitory Effects of BOL-148, Hydrocortisone, and Indomethacin on the Vascular Permeability-increasing Action of ILAPP and Serotonin

Inhibitor	Dose (mg/kg)	Blueing activity (μg of dye/site)	
		ILAPP[a]	Serotonin[b]
BOL-148	0	11±0.9(5)[c]	9±1.4(6)
	0.63	8±0.5(5)*	0±0.1(6)*
Hydrocortisone	0	12±1.5(6)	10±1.3(3)
	50	13±1.4(6)	7±0.5(3)**
Indomethacin	0	13±2.4(3)	9±1.3(3)
	90	16±3.0(3)	6±1.0(3)

a) 0.3 mg/kg.
b) 12 μg/kg.
c) The mean value±S.E. (the number of observations).
* $p<0.01$ and ** $p<0.05$, as compared to the control.

All samples were dissolved in physiological saline at a desired concentration, except that indomethacin was dissolved in physiological saline containing 0.3% triethylamine. BOL-148 was mixed with ILAPP or serotonin, then injected intradermally into the abdomen of mice. Hydrocortisone was given by an intramusclar injection to mice 3 hr before administration of ILAPP or serotonin. Indomethacin was injected intraperitoneally into mice 2.5 hr before administration of ILAPP or serotonin.

of hemorrhagic spots) of trypsin, elastase, Pronase, and subtilisin BPN′ were 5, 7, 16, and 17 mm, respectively. These values are much higher than that of ILAPP, 2 mm. Histamine had no blueing activity at a dose of 3 or 12 μg/kg. The blueing activity of bradykinin was only 3—4 μg of dye/site, even when it was given at high doses (0.1—1 mg/kg).

Table I compares the inhibitory effects of BOL-148, hydrocortisone, and indomethacin on the blueing activities of ILAPP and serotonin. The activity of ILAPP was inhibited by BOL-148 to 73% of the original level, but not by hydrocortisone or indomethacin. The inhibition by BOL-148 was statistically significant relative to the control. The activity of serotonin was completely inhibited by BOL-148 and partially by hydrocortisone and indomethacin.

Table II. Effects of Proteinase Inhibitors on the Vascular Permeability-increasing and Enzymatic Activities of ILAPP

Inhibitor	I/E[a] (molar ratio)	Blueing activity (μg of dye/site)	Caseinolytic activity (%)	Suc-$(Ala)_3$-pNA hydrolytic activity (%)
SBTI	0	13±1.8(6)[b]	100	100
	0.5	5±1.3(6)*	92	91
API	0	12±1.2(6)	100	100
	0.5	3±1.1(6)*	17	1
Pepstatin	0	10±1.3(3)	100	100
	10	10±1.7(3)	110	96
	100	9±2.8(3)	110	111
Leupeptin	0	14±1.4(3)	100	100
	10	9±1.6(3)**	100	99
	100	8±1.6(3)**	98	96

a) Inhibitor/enzyme.
b) The mean value±S.E. (the number of observations).
* $p<0.01$ and ** $p<0.05$, as compared to the control.
ILAPP was preincubated with inhibitors for 30 min at 4° and assayed at a dose of 0.3 mg/kg for blueing activity and at a concentration of 4 μg/ml for the enzymatic activities.

Effects of Proteinase Inhibitors on the Vascular Permeability-increasing and Enzymatic Activities of ILAPP

As shown in Table II, when ILAPP was preincubated with SBTI at a molar ratio of 0.5, the blueing activity of ILAPP was reduced to 38% of the original level, whereas its hydrolytic activities towards casein and Suc-$(Ala)_3$-pNA remained almost at the original level. The hydrolytic activity towards Suc-$(Ala)_3$-pNA was completely inhibited by a ten-fold molar excess of SBTI (data not shown). Leupeptin also inhibited only the blueing activity when the molar ratio of the inhibitor to ILAPP was 10 or 100: 1. All activities were strongly inhibited by API, but pepstatin had no effect.

Vasodilative, Kinin-forming, and Kinin-inactivating Activities of ILAPP

The vasodilative activity of ILAPP was measured at a dose of 0.1, 1, or 10 μg/dog. A slight activity (0.1 KU/mg) was detected only after injection of 10 μg/dog. The kinin-forming activity was assayed after preincubation of 4, 7, or 20 μg of ILAPP with 15 mg of the kininogen, but no activity was detected at any amount.

If ILAPP itself rapidly inactivates kinins produced by the preincubation of the enzyme and kininogen, no kinin-forming activity would be detected. Therefore, the kinin-inactivating activity was measured after preincubation of 4 or 7 μg of ILAPP with 100 ng of bradykinin. The enzyme showed an activity of 3.4 μg of bradykinin inactivated per min per mg. The activities of bromelain, ficin, and papain have been reported to be 3.3, 8.3, and 456 μg, respectively, of bradykinin inactivated per min per mg.[15] The activity of ILAPP thus appears

to be approximately equivalent to that of bromelain.

Esterolytic Activity of ILAPP

The esterolytic activity of ILAPP was determined with derivatives of amino acid methyl esters, one of which, Ac-$(Ala)_3$-OMe, is hydrolyzed by elastase-like enzymes at a high rate and most of which are good substrates for trypsin-like enzymes.

TABLE III. Esterolytic Activity of ILAPP

Substrate	Activity (EU/mg)
Tos–Lys–OMe	0.2
Tos–Arg–OMe	0.6
Ac–Arg–OMe	2.3
Bz–Arg–OMe	3.3
Ac–Gly–Lys–OMe	6.6
Ac–$(Ala)_3$–OMe	8.8

All substrates were incubated with ILAPP at a concentration of 33 mM, except that Ac-$(Ala)_3$-OMe was used at 5 mM.

As shown in Table III, ILAPP hydrolyzed Ac-$(Ala)_3$-OMe at a higher rate than other derivatives of basic amino acid methyl esters. In the case of trypsin, the activity was 82.7 EU/mg for Tos-Arg-OMe and negligible for Ac-$(Ala)_3$-OMe. These results suggest that ILAPP is a type of protease which differs in specificity from trypsin and trypsin-like enzymes.

Discussion

Takahashi and Ohsaka[16] suggested that proteases such as Pronase, subtilisin BPN′, papain, chymotrypsin, and trypsin produced severe necrosis, occasionally followed by atypical hemorrhage induced secondarily at the necrotic lesion, when injected into the back skin of rabbits at a massive dose (50—300 μg/site). In the present study, therefore, the hemorrhage produced to various extents by the proteases tested appeared to be atypical effects on the abdominal skin of mice, though Pronase showed the maximum activity 14 hr after injection, as in the case of Habu toxin, which contains a specific hemorrhagic factor. It has been reported that some proteases, which were separated from heat-injured skin,[17] polymorphonuclear leukocytes,[17] and bone marrow cells,[18] play important roles in the development of inflammations, such as increasing the vascular permeability and leukocyte emigration. The primary effect of these proteases was found to be delayed increase in the vascular permeability, which was produced approximately 4 hr after administration, and appeared to be different from the short-lived increase produced within 30 min. ILAPP had a short-lived increasing effect, as shown in Fig. 1. SBTI and leupeptin both inhibited this effect, whereas they exerted no influence on the hydrolytic activity of ILAPP towards either casein or Suc-$(Ala)_3$-pNA under the same preincubation conditions. API,[19] which was reported to be a strong inhibitor of subtilisin BPN′, inhibited all the activities of ILAPP. BOL-148, which is a specific antagonist to serotonin, was partially inhibitory. These results suggest that the action of ILAPP is

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mediated by several factors including serotonin. One possible explanation for this is that ILAPP activates a protease (s) in various tissues to release a peptide factor (s) into subcellular fractions, and this subsequently increases the vascular permeability. Further work, however, is required to clarify these mechanisms. The vasodilative activity of ILAPP was 0.1 KU/mg. This value is extremely small as compared with that of some kallikreins. For example, hog pancreatic, human urinary, and human salivary kallikreins had activities of 1430 KU/mg, 200, and 1117 KU/E_{280}, respectively.[20] The vasodilative activity has been reported to be usually proportional to the kinin-forming activity.[14] Therefore, ILAPP is not considered to produce kinins from the kininogen by proteolytic action.

In conclusion, the possibility that the insulin-like activity of ILAPP is attributable to kinins produced directly by ILAPP may be excluded.

Acknowledgement A part of this investigation was carried out in the Laboratory of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo. We wish to express our gratitude to Professor C. Moriwaki for his helpful suggestions and we are also grateful to Miss R. Ogata of this Faculty for her technical assistance.

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