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Purification and Characterization of a Novel Serine Proteinase from the Microsomal Fraction of Bovine Pancreas[1]

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A novel trypsin-like serine proteinase was purified to homogeneity from the bovine pancreas microsome fraction. The enzyme was solubilized with 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), and purified by a series of column chromatographic steps on Ultrogel AcA-34, trypsin inhibitor-Sepharose 4B, and arginine-Sepharose 4B. The molecular mass of this pancreas trypsin-like proteinase (bPTLP) was estimated to be 29.5 kDa by SDS-PAGE under reducing conditions. The NH_2-terminal sequence of bPTLP is very homologous, but not identical to those of other serine proteinases, especially such as elastases IV, II, and III. Substrate specificity studies involving a synthetic substrate and glucagon indicated that the enzyme hydrolyzes Arg-X, Lys-X, and Leu-X bonds. The best synthetic substrate for bPTLP was *t*-butyloxycarbonyl Gln-Arg-Arg-4-methylcoumaryl 7-amide. The enzyme failed to hydrolyze the substrate for chymotrypsin and elastase. The enzyme activity was inhibited by diisopropyl fluorophosphate, *p*-amidinophenylmethane sulfonylfluoride, and leupeptin, indicating that it is a serine-proteinase. These findings show that bPTLP is a novel serine-proteinase which differs from all known proteinases. The physiological function of the enzyme has yet to be determined.

Key words: elastase, microsome, pancreas, serine proteinase, trypsin-like proteinase.

Trypsin-like serine proteinases are widespread in nature, and are involved in many biological processes, in proteolytic processing of proteins, digestion, blood coagulation, angiogenesis, inflammation, and fertilization (*1*). They may also be implicated in the pathogeneses of various diseases. Recently, Kido *et al.* purified a trypsin-like serine proteinase from rat bronchiolar epithelial cells and showed that it processes the precursor of a viral fusion glycoprotein to the active form (*2*). Tsuji *et al.* indicated that hepsin, a putative membrane-associated trypsin-like serine proteinase expressed at a high level in liver (*3*), may play a significant role in cell growth and the maintenance of cell morphology (*4*). Kazama *et al.* showed that hepsin is capable of initiating the coagulation pathway on the cell surface that ultimately leads to thrombin formation (*5*). Thus, identification of a novel trypsin-like proteinase could aid our understanding of basic biological processes as well as the pathogeneses of some diseases.

In this study, we purified a novel trypsin-like serine proteinase to homogeneity from a microsomal fraction of bovine pancreas. Its properties, including the amino-terminal amino acid sequence, amino acid composition, substrate specificity and proteinase inhibitor profile, are described.

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Abbreviations: APMSF, amidinophenylmethanesulfonyl fluoride; Bz, benzoyl; Z, benzyloxycarbonyl; bPTLP, bovine pancreas trypsin-like proteinase; Boc, *t*-butyloxycarbonyl; *p*CMB, *p*-chloromercuribenzoate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DFP, diisopropyl fluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MCA, 4-methylcoumaryl 7-amide; pyr, L-pyroglutamyl; Suc, succinyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Materials—Peptide 4-methylcoumaryl 7-amide (MCA) substrates, human glucagon, leupeptin, pepstatin, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane (E-64), and chymostatin were purchased from the Peptide Institute (Osaka). Arginine-Sepharose 4B and activated CH-Sepharose 4B were from Pharmacia (Uppsala, Sweden). Soybean trypsin inhibitor was from Sigma Chemical (St Louis, MO, USA), and coupled to CH-Sepharose 4B according to the instructions of the manufacturer. [1,3-^{3}H]Diisopropyl fluorophosphate (DFP) was from NEN-Dupont (Boston, MA, USA). The other chemicals used were of analytical grade.

Enzyme Assay—Proteinase activity was assayed with Boc-Gln-Arg-Arg-MCA as described (*6*). One unit was defined as the activity that produced 1 nmol of 4-methylcoumaryl 7-amide per min at 37°C. The protein concentration was determined according to the method of Bradford (*7*). Assays were performed using the Bio-Rad protein assay reagent with bovine serum albumin as the standard.

Electrophoresis—Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*8*). The standard proteins employed were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa),

and lyzozyme (14.4 kDa). The proteins were detected by silver staining.

[^3H]*DFP Labeling*—The purified enzyme (50 ng) was incubated in an ice-bath for 30 min in the absence or presence of 5 mM amidinophenylmethanesulfonyl fluoride (APMSF). Thereafter, [^{3}H]DFP (72 kBq) was added, followed by further incubation in the ice-bath for 30 min. The samples were then boiled in 2% SDS in the presence of β-mercaptoethanol and resolved by SDS-PAGE. The gel was soaked in Amplify (Amersham, Buckinghamshire, UK) and dried. Autoradiography was performed at −80°C with Konica X-ray film.

Amino Acid Composition and Sequence Analysis—The purified enzyme (5 μg) was hydrolyzed in 6-N HCl for 24 h at 110°C in an evacuated, sealed tube. The amino acids were determined with a Shimadzu ALC-1000 amino acid analyzer.

For NH_2-terminal sequence analysis, the purified enzyme (5 μg) separated by SDS-PAGE was electroblotted on a PVDF membrane (Immobilon Transfer, 0.45 μm; Millipore) according to the instructions of the manufacturer. Protein bands were detected by staining with amido black. The membrane was destained with 7% acetic acid, washed with water and then dried. The protein band (29.5 kDa) was cut into small pieces and applied to a Shimadzu Protein sequencer (Model PPSQ-10) equipped with an on-line phenylthiohydantoin amino acid analyzer.

Digestion of Glucagon and Analysis of the Digest—The purified enzyme (34 pmol) was incubated with human glucagon (8.6 nmol) in a final volume of 100 μl in 50 mM Tris-HCl buffer, pH 9.0, at 37°C for 1 h. The digestion mixture was acidified with 40 μl of 1% trifluoroacetic acid. Samples were subjected to reversed phase high performance liquid chromatography (HPLC) on a Spherisorb-ODS 2 column (4×100 mm; Pharmacia-LKB). Peptides were eluted at 0.5 ml/min with a linear gradient of acetonitrile (0–50% in 50 min) in 0.1% trifluoroacetic acid. The peaks were collected, applied to glass fiber discs which had been coated with Polybrene, and then analyzed with an automated protein sequencer (Shimadzu PPSQ-10).

RESULTS AND DISCUSSION

Purification of the Proteinase—All purification procedures were performed at 4°C unless otherwise stated. Fresh defatted bovine pancreas (600 g) was minced and homogenized with 2.5 liters of 0.25 M sucrose containing 1 mM Tris-HCl, pH 7.0, in a Waring blender for 1 min. The homogenate was centrifuged at 700×g for 10 min. The supernatant was centrifuged at 10,000×g for 15 min and then filtered through a gauze. The filtrate was further centrifuged at 105,000×g for 30 min. The precipitated microsome membranes were homogenized in a Teflon homogenizer with 4 volumes of 50 mM acetate buffer, pH 6.0, containing 0.5 M NaCl, and then centrifuged at 105,000×g for 1 h. The precipitate was homogenized in the same buffer and then centrifuged again. The microsomal membranes were washed with 0.5 M NaCl, and then suspended in 4 volumes of 50 mM acetate buffer, pH 6.0, containing 1% [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and incubated overnight with stirring. After centrifugation at 105,000×g for 1 h, the supernatant was concentrated by ultrafiltration (Amicon, YM-30). The concentrate (1,500 mg protein) was applied to an Ultrogel AcA-34 column (4.3×93 cm) equilibrated with 25 mM acetate buffer, pH 6.0, containing 0.1% CHAPS and 1 mM $CaCl_2$, and eluted with the same buffer at the flow rate of 50 ml/h. As shown in Fig. 1A, fractions of the main peak (tube numbers 39–54), which corresponded to a protein of about 500 kDa, were pooled. This sample was concentrated by ultrafiltration and then dialyzed against 20 mM Tris-HCl buffer, pH 7.0, containing 0.1% CHAPS. After centrifugation to remove insoluble materials, the supernatant was applied to a trypsin inhibitor-Sepharose 4B column (1.3×1.3 cm) equilibrated with the dialysis buffer. After the column had been washed with the buffer containing 2 M NaCl and with the acetate buffer, pH 4.0, the enzyme activity was eluted with 50 mM glycine-HCl buffer, pH 3.0, at the flow rate of 30 ml/h, as shown in Fig. 1B. The eluate was immediately neutralized with 1 M Tris-HCl buffer, pH 7.0, concentrated by ultrafiltration, and then dialyzed against 20 mM Tris-HCl buffer, pH 7. The dialyzate was applied to an arginine-Sepharose 4B column (1.0×2.5 cm) equilibrated with the same buffer. The enzyme activity was eluted in the 50 and 100 mM NaCl fractions, as shown in Fig. 1C. Both fractions were concen-

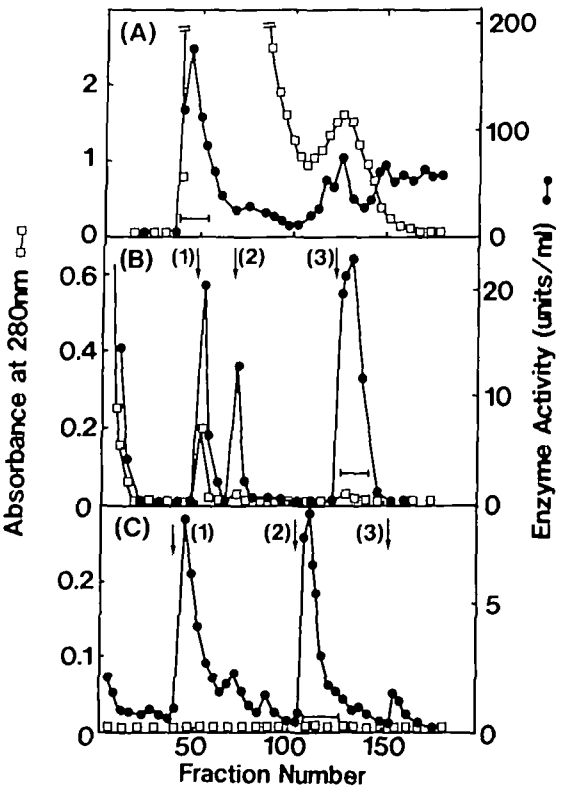

Fig. 1. **Purification of the proteinase from bovine pancreas by a series of column chromatographic steps.** (A) Ultrogel AcA-34 gel filtration. Fractions of 10 ml were collected. (B) Trypsin inhibitor-Sepharose 4B affinity chromatography. The column was eluted with 2 M NaCl (1), 50 mM glycine-HCl buffer, pH 4.0 (2), and 50 mM glycine-HCl buffer, pH 3.0 (3), fractions of 1.5 ml being collected. (C) Arginine-Sepharose 4B affinity chromatography. The column was eluted with 20 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl (1), 100 mM NaCl (2), and 200 mM NaCl (3). Arrows indicate buffer changes. Fractions of 1.5 ml were collected. The fractions indicated by the horizontal bar were pooled.

trated by ultrafiltration and their NH_2-terminal amino acids were analyzed to determine the purity of the enzyme. A single amino-terminal residue (Val), which was proportional to the amount of sample, was detected in the 100 mM NaCl fraction. However, two amino-terminal residues (Val and Ile) were detected in the 50 mM NaCl fraction. We therefore used the 100 mM NaCl fraction as the final preparation with which to characterize the enzyme.

The final preparation gave a single protein band corresponding to a molecular mass of 29.5 kDa on SDS-PAGE, as shown in Fig. 2A. The 29.5 kDa band was labeled with [^{3}H]DFP, as shown in Fig. 2B. The enzyme could not be labeled with [^{3}H]DFP after exposure to APMSF (data not shown). These findings suggested that the enzyme is a serine proteinase. Table I summarizes the results of a typical purification of the enzyme. From 600 g of bovine pancreas, we obtained 29 μg of purified enzyme.

The binding of the purified enzyme to concanavalin and wheat germ agglutinin gels was examined. The enzyme did not bind to these lectin gels (data not shown).

The crude enzyme was eluted at a position corresponding to a molecular mass of about 500 kDa on Ultrogel ACA-34, as shown in Fig. 1A. However, the molecular mass of the purified enzyme was estimated to be 21 kDa on gel filtration on Superdex 200 using the SMART system (Pharmacia Biotech), indicating that it is composed of a single polypeptide chain. The enzyme might be dissociated from other membrane proteins by a high ionic (2 M NaCl) or acidic buffer (pH 3) during the trypsin inhibitor-Sepharose affinity chromatography during the process of purification. Previously we purified a serine-proteinase complexed with α-macroglobulin as a high molecular weight proteinase from BHK cells (*6, 9*) and rat liver (*10*). Uchino *et al.* also purified an α_2-macroglobulin-serine proteinase complex from porcine gastric mucosa (*11*). They showed that the proteinase released from α_2-macroglobulin by acid treatment (pH 3) remained active. To determine whether or not the enzyme is complexed with α_2-macroglobulin, the crude enzyme was immunoblotted and immunoprecipitated with anti-bovine α_2-macroglobulin. The eluate from Ultrogel AcA-34 was used as a crude enzyme and analyzed as described (*9, 10*). However, cross-reactivity with anti-α_2-macroglobulin was not detected and the enzyme activity was not immunoprecipitated (data not shown). These results indicated that the proteinase was not derived from the α_2-macroglobulin-proteinase complex. Further analysis is necessary to determine the cause of the difference in molecular mass between the crude and purified enzymes.

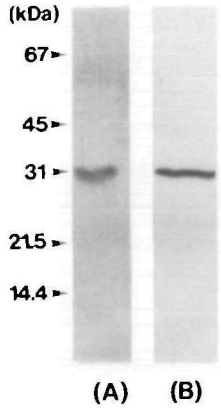

Fig 2 **SDS-PAGE of the purified enzyme.** (A) The purified enzyme (50 ng) was boiled in 2% SDS in the presence of 10% β-mercaptoethanol and then resolved by electrophoresis on a 15% gel. Protein was then detected by silver staining. (B) The purified enzyme was labeled with [^{3}H]DFP as described under "EXPERIMENTAL PROCEDURES," and then resolved by electrophoresis.

TABLE I **Summary of purification of bovine pancreas protease.**

Fraction	Protein (mg)	Activity (units)	Activity (units/mg)	Purification (-fold)
Ultrogel AcA-34	40 3	1,040	25 8	1
Trypsin inhibitor-Sepharose	0 32	236	736	28.5
Arginine-Sepharose	0 029	133	4,570	177

			5 10 15 20
Bovine	pancreas	bPTLP	VVGGEDAIPHSWPWQISLQYLRD
		Trypsin	IVGGREAPGSKWPWQVSLRLKGQ
		Cymotrypsin	IVNGEEAVPGSWPWQVSLQDKTG
	plasma	Plasmin	VVGGCVAHPHSWPWQVSLRTRFG
		Thrombin	IVEGQDAEVGLSPWQVMLFRKSP
Porcine	pancreas	Elastase	VVGGTEAQRNSWPSQISLQYRSG
Rat	pancreas	Elastase I	VVGGAEARRNSWPS ISLQYLSG
		Elastase II	VVGGQEASPNSWPWQVSLQYLSS
		Elastase III	VVHGEDAVPYSWPWQVSLQYEKS
		Elastase IV	VVGGEDAVPNSWAWQVSLQYLKD
	submandibular gland	Kallikrein	VVGGYNCETNSQPWQVAVIGTTF
Human	pancreas	Elastase IIIA	VVHGEDAVPYSWPWQVSLQYEKS
	plasma	Kallikrein	IVGGTNSSWGEWPWQVSLQVKLT
	leucocyte	Elastase	IVGGRRARPHAWPFMVSLQLRGG

Fig. 3. **Amino-terminal sequence of the purified enzyme (bPTLP), and comparison of the sequence with those of the catalytic chains of a variety of other serine proteinases within the trypsin superfamily, comprising bovine trypsin (*12*), chymotrypsin (*13*), plasmin (*14*), and thrombin (*15*); porcine pancreatic elastase (*16*); rat pancreatic elastases I (*17*), II (*17*), III (*18*), and IV (*19*), and tissue kallikrein (*20*); and human pancreatic elastase IIIA (*21*), plasma kallikrein (*22*), and leucocyte elastase (*23*).** Residues identical to those in the proteinase are shaded.

NH_2-Terminal Sequence and Amino Acid Composition—A single amino-terminal sequence of the enzyme was obtained, as shown in Fig. 3. This sequence was not found in the SWISS-PROT, NBRF, and PRF/SEQDB databases. Figure 3 shows a comparison of the NH_2-terminal sequence of the enzyme with those of various bovine, porcine, rat, and human serine-proteinases. The enzyme (bPTLP) shares 35-78% identity with these proteinases. Interestingly, the NH_2-terminal sequence of the enzyme was highly similar to those of pancreatic elastases regardless of the animal species. Its identities with porcine pancreatic elastase, rat pancreatic elastases I, II, III, and IV, and human pancreatic elastase IIIA are 61, 61, 70, 70, 78, and 70%, respectively. However its identity with human leucocyte elastase is not so high (39%).

On the contrary, the amino acid composition of the enzyme was quite different from those of pancreatic elastases II and IV (Table II). The contents of glutamic acid, glycine, valine, phenylalanine, and histidine clearly differed.

Optimum pH and pH Stability—The effects of pH on the activity toward Boc-Gln-Arg-Arg-MCA and on the stability of the enzyme were examined. The enzyme showed the highest activity at pH 9.0, as shown in Fig. 4A. However, it exhibited significant activity at pH 7.4 (85% of maximum).

On the other hand, the enzyme was more stable at acidic than basic pH, as shown in Fig. 4B. When the enzyme was incubated at 37°C for 10 min at pH 7.0, 93% of its activity was lost, indicating that it is extremely labile. The profiles of the pH dependence of the activity and stability of the enzyme revealed a reverse correlation.

Substrate Specificity toward Various Synthetic Substrates—Table III shows the activities of the purified enzyme toward various synthetic substrates. The enzyme was most active toward Boc-Gln-Arg-Arg-MCA among these substrates. This substrate was judged to be the most suitable from the k_{cat}/K_m value (Table IV). The enzyme hydrolyzed substrates with a basic amino acid at the P1 position. On the contrary, substrates with single or double amino acid residues, such as Bz-Arg-MCA, Z-Phe-Arg-MCA, and Z-Arg-Arg-MCA, which are cleaved by trypsin, were hardly hydrolyzed by the enzyme. This suggested that P_3 residues in the substrate are important in determining

TABLE II. **Amino acid composition of the purified enzyme.**

Amino acid	bPTLP	Pancreatic elastase	
		II[a]	IV[b]
		(mol%)	
Aspartic acid	8.4	9.7	9.6
Threonine	4.3	6.2	7.5
Serine	10.8	13.2	8.8
Glutamic acid	15.1	6.2	8.4
Proline	2.3	4.4	4.6
Glycine	19.0	10.6	9.6
Alanine	6.9	6.6	6.3
Cysteine	ND	3.1	4.6
Valine	4.4	10.0	8.8
Methionine	ND	0.4	0.8
Isoleucine	3.6	3.5	5.9
Leucine	5.9	9.3	7.1
Tyrosine	1.7	3.1	2.5
Phenylalanine	3.1	0.9	1.7
Histidine	6.3	2.2	2.5
Lysine	4.4	4.0	3.8
Tryptophan	ND	3.5	4.2
Arginine	3.9	3.1	3.4

ND, not determined. [a,b]Mature form of elastase II (*17*) and elastase IV (*19*).

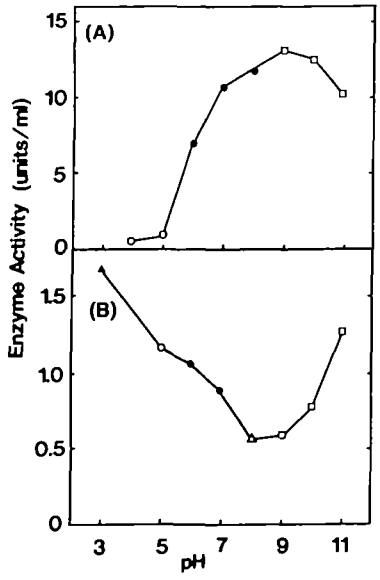

Fig. 4. **Effects of pH on the activity and stability of the enzyme.** (A) The pH optimum for the enzyme activity was determined in buffers of various pHs. Assays were performed under the standard conditions except for the buffers used. (B) The pH dependence of the enzyme stability. The purified enzyme was incubated in buffers of various pHs for 30 min in an ice-bath, and then the activities were assayed under the standard conditions. These buffers included glycine-HCl (closed triangle), acetate (open circles), sodium phosphate (closed circles), Tris-HCl (open triangle), and glycine-NaOH (open squares).

TABLE III. **Substrate specificity of the purified enzyme.** The enzyme activity was determined in 0.1 M Tris-HCl buffer, pH 9.0, with various substrates at a concentration of 10 μM.

Substrate	Activity (units/ml)	Relative activity (%)
Boc-Gln-Arg-Arg-MCA	22.5	100
Boc-Val-Pro-Arg-MCA	11.6	52
Boc-Leu-Lys-Arg-MCA	4.86	22
Boc-Phe-Ser-Arg-MCA	4.13	18
Boc-Glu-Lys-Lys-MCA	3.50	16
pyr-Arg-Thr-Lys-Arg-MCA	1.71	8
Boc-Ile-Glu-Gly-Arg-MCA	1.32	6
Boc-Arg-Val-Arg-Arg-MCA	0.71	3
Boc-Val-Leu-Lys-MCA	0.15	0.7
Z-Phe-Arg-MCA	0.42	2
Z-Arg-Arg-MCA	0.17	0.8
Bz-Arg-MCA	0.02	0.1
Suc-Ala-Pro-Ala-MCA	0.02	0.1
Suc-Leu-Leu-Val-Tyr-MCA	0.03	0.1

TABLE IV. **Kinetic parameters of the purified enzyme.** The k_{cat} values were calculated assuming that 29.5 mg of protein represents 1 μmol of enzyme.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
Boc-Gln-Arg-Arg-MCA	25.7	0.667	38.5
Boc-Phe-Ser-Arg-MCA	1.00	0.149	6.71
Boc-Leu-Lys-Arg-MCA	1.10	0.400	2.75

the catalytic efficiency of the enzyme. The enzyme did not hydrolyze Suc-Leu-Leu-Val-Tyr-MCA, which is a good substrate for chymotrypsin (*24*). Although sequence analysis showed that the enzyme is homologous to pancreatic elastase, it did not hydrolyze Suc-Ala-Pro-Ala-MCA, which is a good substrate for elastase (*25*). Tamanoue *et al.* purified a 32 kDa serine proteinase from microsome membranes of rat liver and reported that its optimal synthetic substrate was Boc-Gln-Arg-Arg-MCA, like in the case of our purified enzyme (*26*). However, the activities of the rat liver enzyme toward other synthetic substrates, such as Boc-Phe-Ser-Arg-MCA, Boc-Val-Pro-Arg-MCA, and Boc-Glu-Lys-Lys-MCA, were low compared with those of our purified enzyme.

Hydrolysis of Glucagon—The ability of the enzyme to hydrolyze glucagon was examined. Cleavage of glucagon yielded five peptides which were separated by reverse-phase HPLC (Fig. 5). Then their amino acid sequences were analyzed. About the same molar amounts of products was obtained except for that of peptide 3. Cleavage occurred on the carboxyl side of lysine, arginine and leucine (Leu^{26}) residues. It is interesting to note the cleavage of glucagon at the Leu-Met bonds. So far it is known that chymotrypsin C (*27*), chymase (*28*), elastase (*29*), and cathepsin G (*30*) can cleave Leu-X bonds among the trypsin superfamily. The enzyme did not cleave Suc-Leu-Leu-Val-Tyr-MCA (a good substrate for chymotrypsin, cathepsin G and chymase) and glucagon was not cleaved at phenylalanine[6,22] or tyrosine[10,13] residues. Moreover, the proteinase (bPTLP) failed to cleave Suc-Ala-Pro-Ala-MCA (a good substrate for elastase). These results indicated that the proteinase is free from chymotrypsin, chymase, cathepsin G, and elastase activities. Although there are two leucine (Leu^{14} and Leu^{26}) residues in glucagon, only the carboxyl side of Leu^{26} was hydrolyzed. As suggested by the finding that tripeptide-MCA substrates, such as Boc-Gln-Arg-Arg-MCA and Boc-Val-Pro-Arg-MCA, but not dipeptide-MCA substrates, such as Z-Phe-Arg-MCA and Z-Arg-Arg-MCA, are good substrates, the filling of at least four binding sites (S_3, S_2, S_1, and S_1') seems to be a prerequisite for hydrolysis. If the cleavage of glucagon on the carboxy-terminal side of Lys^{12} and Arg^{17} occurred preferentially, the cleavage on the carboxyl side of Leu^{14} would not occur because a P3 residue (Lys^{12}) is not present in the substrate and the enzyme has no exopeptidase activity.

On the contrary, glucagon has one paired arginine site (Arg^{17}-Arg^{18}). The enzyme cleaved on the carboxy-terminal side of Arg^{18}, but predominantly between Arg^{17} and Arg^{18}. The extent of the cleavage is 12 and 88%, respectively. Further systematic kinetic studies and determination of the primary structure of the enzyme (bPTLP) are necessary to clarify the specificity of the substrate binding site.

Effects of Inhibitors—The effects of various inhibitors on the activity were examined (Table V). DFP, APMSF, and leupeptin inhibited the activity, whereas chymostatin, E-64, pepstatin A, and EDTA had no effect on the activity. These results indicated that the enzyme is a trypsin-like serine proteinase. The proteinase purified from the microsomal membranes of rat liver is partially inhibited by E-64 and *o*-phenanthroline (*11*). However, these compounds had no effect on the activity of the bovine pancreas enzyme. These results confirmed that the bovine pancreas enzyme is distinct from the rat liver enzyme purified by Tamanoue *et al.* (*26*). The activity of the bovine pancreas enzyme was inhibited by *p*-chloromercuric benzoate (*p*CMB), although E-64, a specific cysteine proteinase inhibitor, had no effect. The inhibitory effect of *p*CMB may be due to the presence of a heavy metal ion (Hg^{2+}) in this reagent.

A trypsin-like serine proteinase has been isolated as a likely proenkephalin processing proteinase, from chromaffin granule membranes, by Shen *et al.* (*31*). Its molecular mass, pH optimum, profile of susceptibility to inhibitors, and cleavage specificity towards peptide substrates were largely similar to those of the pancreas enzyme. However, the chromaffin enzyme was not inhibited by *p*CMB and did not cleave Leu-X bonds.

We report here the purification and characterization of a trypsin-like serine proteinase from the microsomal frac-

(A)

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr

(B)

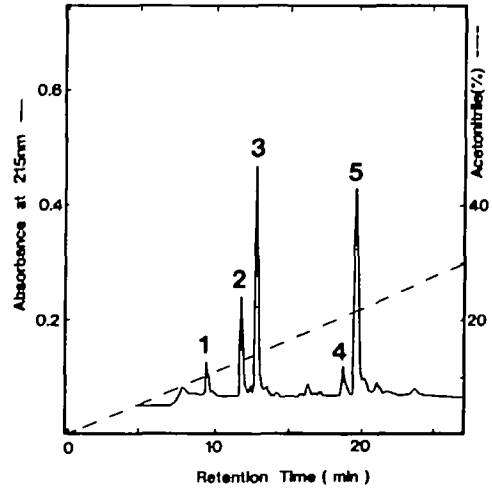

Fig. 5. **Cleavage of glucagon by the proteinase.** (A) Cleavage site of glucagon. Closed and open arrowheads indicate major and minor cleavage sites, respectively. (B) HPLC profile of the digestion mixture of glucagon with the proteinase. The peaks (1-5) were collected and the sequences were determined. The amino acid sequences of the peptides are shown in a one-letter code. 1, MNT; 2, YLDSR; 3, HSQGTFTSDYSK; 4, AQDFVQWL; and 5, RAQDFVQWL. The other peaks were also analyzed, however, no phenylthiohydantoin amino acids were detected.

TABLE V. **Inhibitor sensitivity of the purified enzyme.**

Addition	Concentration	Activity (units/ml)	Inhibition (%)
None		27.9	0
DFP	1 mM	3.90	86
APMSF	5 mM	0.47	98
Leupeptin	2 μM	1.54	94
Chymostatin	2 μM	26.7	4
E-64	3 μM	29.5	0
*p*CMB	1 mM	5.70	80
Pepstatin A	2 μM	26.4	5
EDTA	1 mM	27.5	1
o-Phenanthroline	1 mM	27.7	1

tion of bovine pancreas. The partial amino-terminal sequence is highly homologous, but not identical, with that of pancreatic elastase. Moreover, its amino acid composition and substrate specificity were clearly distinct from those of pancreatic elastase. More recently, Tsuchiya *et al.* purified a novel membrane-bound serine proteinase from porcine intestinal mucosa (*32*). Its molecular weight (32 kDa) estimated by SDS-PAGE under reducing conditions was similar to that of our purified enzyme. However, the substrate specificities toward synthetic substrates and natural peptides of the porcine enzyme differed from those of our purified enzyme. The porcine enzyme is highly specific for the carboxyl side of arginine residues. These results indicated that the proteinase purified from the microsomal fraction of bovine pancreas is a novel proteinase which differs from all known proteinases. The physiological function of the enzyme remains unknown. To determine the role of the enzyme, it is necessary to examine its cellular and intracellular localization. For this purpose immunohistochemical studies on the cell distribution of the enzyme are currently in progress.

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