J. Biochem. 130, 425–430 (2001)

Characterization of a Membrane-Bound Arginine-Specific Serine Protease from Rat Intestinal Mucosa[1]

Kenji Kishi,* Kazuya Yamazaki,* Ikuko Yasuda,* Naohisa Yahagi,† Masao Ichinose,† Yuichi Tsuchiya,‡ Senarath B.P. Athauda,*,§ Hideshi Inoue,* and Kenji Takahashi*,[2]

*School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392; †Department of Internal Medicine, Faculty of Medicine, The University of Tokyo, Tokyo 113-8655; ‡Department of Biochemistry, Toho University School of Medicine, Tokyo 143-8540; and §Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka

Received March 26, 2001; accepted June 25, 2001

Previously we isolated and characterized a membrane-bound, arginine-specific serine protease from pig intestinal mucosa [*J. Biol. Chem.* 269, 32985–32991 (1994)]. For further characterization of this type of enzyme, we cloned a cDNA from rat intestinal mucosa encoding the precursor of a similar protease. The partial amino acid sequences determined for the pig enzyme were found to be shared almost completely by the rat enzyme. The serine protease domain of the rat enzyme, heterologously expressed in *Escherichia coli*, specifically cleaved Arg (or Lys)-X bonds with a marked preference for Arg-Arg or Arg-Lys, similar to the pig enzyme. The mRNA for the rat enzyme was shown to be distributed mainly in intestine, and the enzyme was detected in the duodenal mucosa as a 70 kDa protein. Immunohistochemical analysis of the small intestinal tissue showed that the enzyme is localized mainly on brushborder membranes.

Key words: brushborder membrane, intestinal mucosa, serine protease, subcellular localization, substrate specificity.

Protein digestion in the intestine is carried out by several proteases, and is also regulated by various kinds of gastrointestinal polypeptides. Previously, we purified a membrane-bound serine protease from pig intestinal mucosa, which digested proteins such as histone, lysozyme, γ-globulin, casein, and bovine serum albumin only slightly, but cleaved several peptide 4-methylcoumaryl-7-amide (MCA) substrates at arginine residues, and also certain neuropeptides at arginine residues, especially between pairs of basic amino acids, Arg-Arg or Arg-Lys (*1*). This distinct characteristic suggested the possibility that this enzyme might be involved as a processing protease in the generation of certain gastrointestinal neuropeptides or peptide hormones from their precursors, or their specific degradation.

In this study, molecular cloning of the cDNA for the rat membrane-bound serine protease was performed to provide further characterization of this type of protease. The clone thus obtained was found to encode a mosaic protein of 855 amino acid residues, comprising various non-catalytic domains followed by a serine protease domain as in the case of enteropeptidase (*2–4*). Moreover, the same or very similar amino acid sequences were shown to be shared between the rat and pig enzymes by comparison of the rat sequence with partial sequences of the pig enzyme. The serine protease domain of the rat enzyme expressed in *Escherichia coli* showed similar substrate specificity and susceptibility to various reagents to those of the pig enzyme. Furthermore, the tissue distribution and subcellular localization of the rat enzyme, investigated by RNA blot and immunohistochemical analyses, respectively, demonstrated that the enzyme is distributed mainly in intestinal brushborder membranes. These results indicate that the rat enzyme is essentially the same as the pig enzyme from the intestinal mucosa and may play some specific role(s), mainly on brushborder membranes.

The occurrence of similar membrane-bound serine proteases has been reported recently, including those from mouse thymic stromal cells, named "epithin" (*5*), from human breast cancer cells, named "matriptase" (*6*) and from human epithelial cancer and normal tissues, called "membrane-type serine protease 1 (MT-SP1)" (*7*). These proteases have the same mosaic structure and high sequence identities with the rat enzyme, indicating that they are orthologs of the rat and pig enzymes, although their physiological roles may vary in different cells or tissues.

MATERIALS AND METHODS

RNA Isolation and cDNA Synthesis—Messenger RNA was purified from the duodenum of Wistar strain male rats (7 weeks old, Japan SLC). Duodenum was excised, washed with ice-cold phosphate-buffered saline and kept frozen in liquid nitrogen. Total RNA was prepared from the tissue according to the guanidium thiocyanate-phenol-chloroform

[1] This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 07458149 and 0948015) from the Ministry of Education, Science, Sports and Culture of Japan. The nucleotide sequence for the amino acid sequence of the rat enzyme precursor reported in this paper has been submitted to the GenBank™/EMBL with accession number AB049189.

[2] To whom correspondence should be addressed. Phone: +81-426-76-7146, Fax: +81-426-76-7149, E-mail: kenjitak@ls.toyaku.ac.jp

Abbreviations: MCA, 4-methylcoumaryl-7-amide; MT-SP1, membrane-type serine protease 1.

extraction method (*8*). Poly(A)$^+$RNA was selected by a QuickPrep® Micro mRNA purification kit (Amersham Pharmacia Biotech.). Double stranded cDNA was synthesized using a TimeSaver™ cDNA synthesis kit (Amersham Pharmacia Biotech.) and a Marathon™ cDNA amplification kit (CLONTECH).

cDNA Cloning—BLAST search of the database using the partial amino acid sequences of pig membrane-bound arginine specific serine protease (see Fig. 1) revealed some EST clones from human and mouse. One of the human EST clones was obtained from I.M.A.G.E. Consortium Clone (ID 650505, GeneBank accesion number AA219372) (*9*), and its complete nucleotide sequence was determined. Based on the sequence, DNA primers were designed: GACTCAGTGCTGAGCCTCAC as a sense primer, and GAGTGGAAGGTCAGGTTGTAG and CAGTGTGATGAGCAGGACG as antisense primers. Using these primers, the cDNAs synthesized by the TimeSaver™ cDNA synthesis kit, DNA polymerase r*Taq* (Toyobo, Osaka) and a thermal cycler, GeneAmp 2400 (Perkin-Elmer Biosystems), the polymerase chain reaction (PCR) was performed. The sequence of the amplified DNA was analyzed, and several DNA primers specific for the sequence were designed. Using cDNAs synthesized by the Marathon™ cDNA amplification kit, the DNA primers, the adapter DNA, and Advantage Polymerase Mix (CLONTECH), 5′- and 3′-RACE PCRs were performed.

Expression of the Serine Protease Domain in E. coli—The polypeptide of residues 596–855 (see Fig. 1), including the serine protease domain (residues 615–855), was expressed in *E. coli* under the control of the T7 promoter as follows. Using a sense primer, GGATCATATGGGCTCGGATGAGAAAAACTG, and an antisense primer, TTATTGAATTCTACCCCAGTTTGCTCTTTGATCC, the region of the cDNA encoding residues 596–855 was amplified by PCR. The amplified DNA was digested with *Nde*I and *Eco*RI, and then ligated into a T7 expression plasmid vector, pT7-7. *E. coli* BL21-CodonPlus™(DE3)-RIL (Stratagene) was transformed with the resulting plasmid. The transformed cells were cultured in M9ZB broth in the presence of 0.05 mg/ml ampicillin (*10*). When the absorbance at 600 nm reached 0.6–1.0, the expression of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). The cells were further incubated at 37°C for 150 min with shaking, then harvested by centrifugation and suspended in 50 mM Tris/HCl (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. To this was added lysozyme to a final concentration of 1 mg/ml, and the mixture was kept on ice for 30 min. The resulting solution was subjected to sonication (100 W for 20 s, repeated 6 times) and then centrifuged at 17,000 ×*g* for 10 min at 4°C. After removing the supernatant, the pellet was dissolved in 50 ml of 50 mM Tris/HCl, pH 7.0, containing 1 mM EDTA, 8 M urea, and 30 mM 2-mercaptoethanol. The solution was left to stand at room temperature for 1 h, and then centrifuged at 100,000 ×*g* for 30 min. The supernatant was dialyzed successively against 50 mM Tris/HCl, pH 7.0, containing 1 mM EDTA, 4 M urea, and 30 mM 2-mercaptoethanol for 1 h, against 50 mM Tris/HCl, pH 7.0, containing 1 mM EDTA, 2 M urea, and 30 mM 2-mercaptoethanol for 1 h, against 50 mM Tris/HCl, pH 7.0, containing 1 mM EDTA and 30 mM 2-mercaptoethanol for 1 h, and against 50 mM Tris/HCl, pH 7.0, containing 1 mM EDTA. The sample solution was then incubated at 37°C for 2 h for activation. The dialyzed solution was applied to a column of benzamidine-Sepharose (Amersham Pharmacia Biotech.) and eluted with 50 mM benzamidine. The active fractions were pooled and desalted on a PD-10 column (Amersham Pharmacia Biotech.).

Enzyme Assay and Digestion of Peptides—Enzyme assays toward MCA substrates (Peptide Institute, Osaka) were performed at pH 9.5 and 37°C with fluorometric detection. The peptides were then digested at pH 8.0 and 37°C for 2.5 h followed by HPLC analysis of the digestion products essentially as described (*1*).

Expression in Cultured Cells—The region of cDNA from 6 bases upstream of the initiation codon to the C-terminal end was amplified by PCR using a sense primer, GGATGAATTCGGGAGACCATGGGGAAC, and an antisense primer, GGATGAATTCCTACCCCAGTTTGCTCTTTG. The PCR product was digested with *Eco*RI and inserted into the *Eco*RI site of the expression vector pcDNA3.1/Myc-His(+) A (Invitrogen). The nucleotide sequence of the inserted DNA was confirmed using a DNA sequencer, ABI PRISM™ 377 (Applied Biosystems). The resulting plasmid was purified with an EndoFree™ Plasmid kit (QIAGEN) and used to transfect cultured cells. Transfection of COS-7 cells was performed using LIPOFECT AMIN PLUS™ Reagent (GIBCO BRL) according to the manufacturer's protocol. The transfected COS-7 cells were cultured in Minimum Essential Medium (GIBCO BRL) containing 20% fetal bovine serum at 37°C for 24–48 h.

Western Blot Analysis of Whole Extracts of Rat Intestinal Mucosa—Duodenum was obtained from a Wistar strain male rat (7 weeks old, Japan SLC), and washed with potassium phosphate–buffered saline. The mucosal cells were peeled off with a spatula and collected. The cells were washed with phosphate-buffered saline and suspended in Tris/HCl buffer (pH 6.8). Electrophoresis was performed in a 7.5% SDS–polyacrylamide gel, and then the protein in the gel was transferred to a polyvinylidene difluoride membrane. Immunostaining was performed with a rabbit antibody raised against the recombinant enzyme using a peroxidase-labeled secondary antibody and a VECTASTAIN® ABC kit (Vector Laboratories).

Immunohistochemical Studies—Tissue samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin and cut into serial sections. One was stained with hematoxilin-eosin for routine histological assessment. The avidin-biotin-peroxidase complex method was also used to determine the subcellular localization of the enzyme. Rabbit antibodies raised against the recombinant enzyme, affinity-purified biotin-labeled goat anti-rabbit IgG and the VECTASTAIN® ABC kit were applied to the immunohistochemical demonstration of binding using established methods.

RESULTS

cDNA and Deduced Amino Acid Sequence—A cDNA coding for a rat membrane-bound serine protease was obtained as described in "MATERIALS AND METHODS." It is 3,173 bp long with an open reading frame coding for 855 amino acids (Fig. 1). This protease has a mosaic structure composed of a transmembrane domain, two CUB (complement factor 1R-urchin embryonic growth factor–bone morphoge-

netic protein) domains, four LDLR (low density lipo-protein receptor) domains, and a serine protease domain, aligned in this order. All of the partial sequences previously determined with the membrane-bound, arginine-specific serine protease from pig intestinal mucosa (*1*) were found to be identical or almost identical to the corresponding sequences in the serine protease domain of the rat enzyme (Fig. 1). Among the 52 residues compared, 43 residues (83%) were identical between the two enzymes.

Substrate Specificity and Effects of Inhibitors—The serine protease domain preceded by the C-terminal 19-residue segment of the non-catalytic domain was expressed in *E. coli* as inclusion bodies. From the inclusion bodies, the recombinant protein was extracted with buffer containing 8 M urea and 2-mercaptoethanol, and then refolded by removing the reagents. The recombinant protein was partially activated during the refolding process and fully activated by further incubation at 37°C. Similar autoactivation has been observed for the human enzyme (*7*). The refolded active form was purified on a column of benzamidine-Sepharose and gave a single band on SDS-PAGE (data not shown).

The enzymatic activities of the recombinant serine protease domain toward various peptide MCA substrates were assayed. One milligram of the recombinant enzyme cleaved 2.4 μmol of Boc-Gln-Ala-Arg-MCA per minute. Although this specific activity is lower than that reported previously for the pig enzyme (*1*), the substrate preference of the recombinant protease domain was similar to that of the pig enzyme; Arg was preferred at the P1 site (Table I). The re-

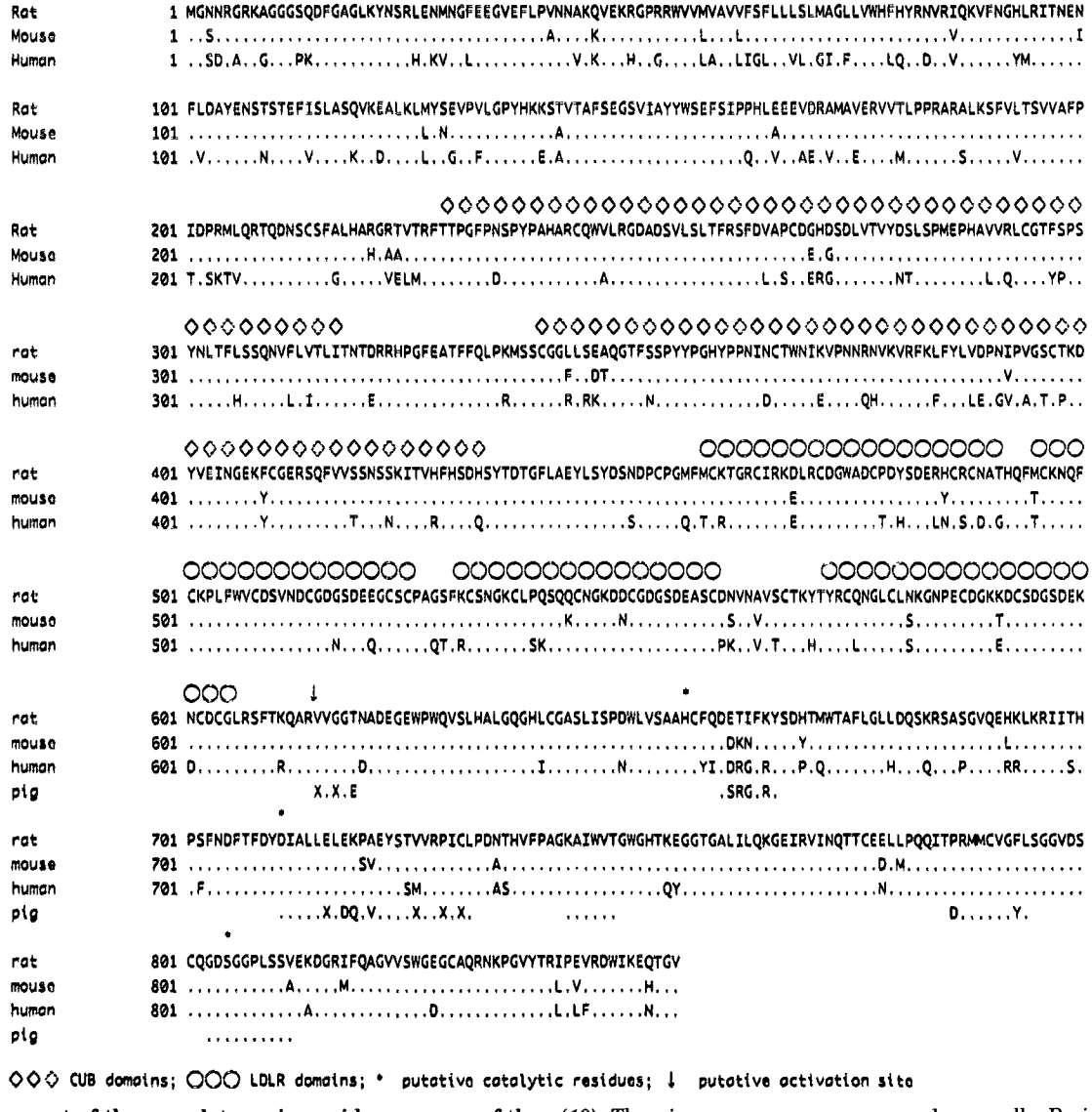

Fig. 1. **Alignment of the complete amino acid sequences of the precursors of the rat membrane-bound, arginine-specific serine protease, mouse epithin (*5*), human matriptase/MT-SP1 (*6*, *7*), and the partial sequences of pig intestine membrane-bound arginine-specific serine protease (*1*).** The alignment of the rat, mouse, and human enzymes was generated using Clustal W1.7 (*13*). The pig sequences were arranged manually. Residues in the mouse, human, and pig enzymes identical to those in the rat enzyme are indicated by dots. XVXGE is the N-terminus of the serine protease domain of the pig enzyme. Parts of the pig sequence, XVXGE, DRMMCVGYL, and DSGGPLSXVE, were reported previously (*1*). X represents an unidentified residue.

combinant enzyme also cleaved certain neuropeptides efficiently (Fig. 2). The cleavage site was restricted mainly to the peptide bond between two consecutive basic residues, Arg-Arg and Arg-Lys. The recombinant enzyme was strongly inhibited by serine protease inhibitors, and partially inhibited by *o*-phenanthroline, as in the case of the pig enzyme (Table II).

Tissue Distribution—Northern blot analysis of the total RNAs from various rat tissues indicated a low but significant expression in esophagus and duodenum (Fig. 3). A very weak signal was also observed for RNA from the ileum, jejunum, colon, and kidney. The size of the mRNA detected was estimated to be about 3.5 kb, which is consistent with the length (about 3.2 kb) of the cloned cDNA. The results of RNA blot analysis using Human RNA Master Blot™ also indicated low but significant expression in colon, small intestine, and trachea, and very weak signals in prostate, salivary gland, kidney, appendix, lung, thyroid gland, pancreas, pituitary gland, placenta, fetal kidney, fetal thymus, and stomach (data not shown). Mouse epithin RNA was reported to be detected with RNA from intestine, kidney, and lung, but not brain, heart, liver, testis, and skeletal muscle (*5*). Human MT-SP 1 mRNA has been reported to be expressed in prostate, kidney, lung, small intestine, stomach, colon, placenta, spleen, liver, leukocytes, and thymus, but not in muscle, brain, ovary, and testis (*7*). Although these data are not in full agreement with one another in detail, they consistently indicate expression in the intestine. The content of the specific mRNA was estimated to be 0.001% of the total mRNA from the small intestine.

Expression in Cultured Cells—The full-length protease precursor was expressed as a fusion protein with a *myc* epitope and a histidine tag in COS-7 cells using an expression plasmid, pcDNA3.1. Upon Western blot analysis of whole cell extracts using specific antibodies raised against the recombinant serine protease domain, protein bands of 99 and 110 kDa were detected under both reducing and non-reducing conditions (Fig. 4A). The 99 kDa protein is consistent in molecular size with the complete recombinant fusion protein (M_r 98K). The 110 kDa protein was assumed to be a glycosylated form. No processed form was observed in this system.

TABLE II. **Effects of various reagents on the activity.** The enzyme (0.1 unit) was preincubated with each reagent in 0.1 M glycine-NaOH buffer, pH 9.5, for 30 min at room temperature. The remaining activity was assayed by incubation with Boc-Gln-Ala-Arg-MCA as described in the legend to Table I.

Reagent	Concentration	Rat	Pig (1)
Bovine pancreas trypsin inhibitor	0.2 mg/ml	97	99
Leupeptin	0.1 mM	98	91
EDTA	10 mM	14	0
o-Phenanthroline	10 mM	46	20
Pepstatin A	0.1 mM	0	0

Dynorphin A (1-13) 100%
Tyr-Gly-Gly-Phe-Leu-**Arg** ↓ **Arg**-Ile-Arg-Pro-Lys-Leu-Lys

Neurotensin 83%
<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-**Arg** ↓ **Arg**-Pro-Tyr-Ile-Leu

α-Neoendorphin 66%
Tyr-Gly-Gly-Phe-Leu-**Arg** ↓ Lys-Tyr-Pro-Lys

Oxidized Insulin B chain
Phe-Val-Asn-Gln-His-Leu-Cta-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cta-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (no digestion)

Fig. 2. **Activity of the rat enzyme toward peptide substrates.** The site and extent of cleavage of each peptide (Peptide Institute, Osaka) by the enzyme is shown. The arrow indicates the cleavage site. The extent of cleavage in percent under the specified conditions is shown above each arrow. Each peptide at 1% (w/v) was incubated with the enzyme (0.1 unit) at pH 8.0 and 37°C for 2.5 h as described under "MATERIALS AND METHODS." <Glu, pyroglutamic acid; Cta, cysteic acid.

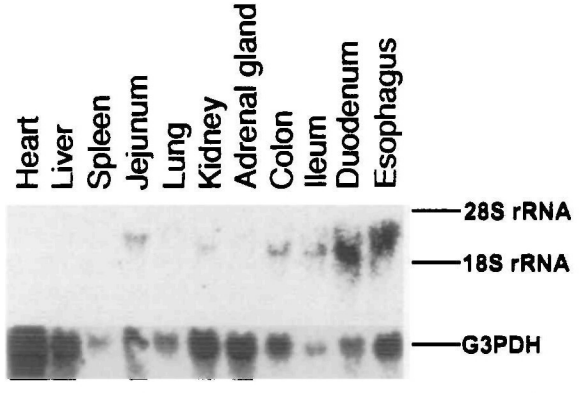

Fig. 3. **Northern blot analysis of the total cellular RNA from various rat organs.** Total cellular RNAs from the rat heart, liver, spleen, jejunum, lung, kidney, adrenal gland, colon, ileum, duodenum, and esophagus (20 μg each) were used. The positions of the 28S and 18S ribosomal RNAs are indicated on the right. Rehybridization of the filter with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA is shown at the bottom.

TABLE I. **Activity of the rat recombinant serine protease domain toward MCA substrates.** Each MCA substrate was incubated with 0.1 unit of enzyme in 0.1 M glycine-NaOH buffer, pH 9.5, for 10 min at 37°C. The activity toward Boc-Gln-Ala-Arg-MCA was taken as 100%, and the relative activities are shown.

Substrate	Rat	Pig (1)	Substrate	Rat	Pig (1)
Boc-Gln-Gly-Arg-MCA	121	77	Boc-Leu-Arg-Arg-MCA	4	nd[a]
Boc-Gln-Ala-Arg-MCA	100	100	Boc-Gly-Arg-Arg-MCA	2	6
Boc-Glu-Lys-Lys-MCA	18	1	Boc-Arg-Val-Arg-Arg-MCA	1	nd
Boc-Leu-Gly-Arg-MCA	17	57	Bz-Arg-MCA	1	0
Boc-Gln-Arg-Arg-MCA	10	45	Z-Arg-Arg-MCA	0	2
Boc-Phe-Ser-Arg-MCA	8	61	Suc-Ala-Ala-Pro-Phe-MCA	0	0
Boc-Leu-Thr-Arg-MCA	7	30	Suc-Leu-Leu-Val-Tyr-MCA	0	0
Boc-Val-Leu-Lys-MCA	6	5	Ala-MCA	0	0
Boc-Leu-Lys-Arg-MCA	5	23			

[a]nd, not determined.

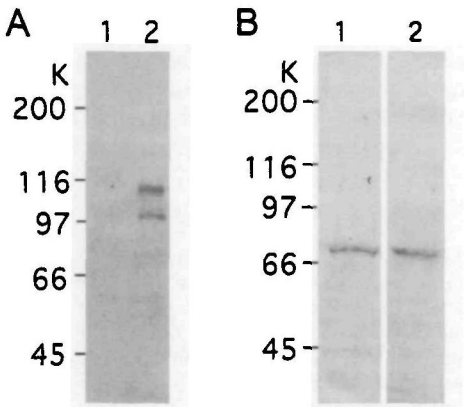

Fig. 4. **Western blot of the rat enzyme.** Panel A: The enzyme expressed in transfected COS-7 cells, after electrophoresis under reducing conditions. Lane 1, un-transfected cells; lane 2, transfected cells. Panel B: The enzyme in the extracts of rat duodenal mucosal cells. Lane 1, after electrophoresis under reducing conditions; lane 2, after electrophoresis under non-reducing conditions.

Western Blot Analysis of Whole Rat Intestinal Mucosa-Extracts—The whole extract from rat intestinal mucosa was submitted to Western blot analysis. Under both reducing and non-reducing conditions, only a single 70 kDa band was detected (Fig. 4B).

Subcellular Localization of the Enzyme—In order to investigate the subcellular localization of the enzyme, immunohistochemical staining was performed using specific antibodies. In rat small intestine, epithelial cells were stained weakly with the specific antibodies with intense staining observed on the brushborder membranes (Fig. 5). There was a regional difference in the intensity of the staining in the intestine; the epithelia stained more strongly in the order of duodenum, jejunum, ileum, and colon, indicating a head to tail gradient in the expression of the enzyme. These results clearly indicate that the enzyme is localized mainly on the brushborder membranes of the intestinal epithelia, with trace amounts also present in the cytosol and on the basolateral membranes.

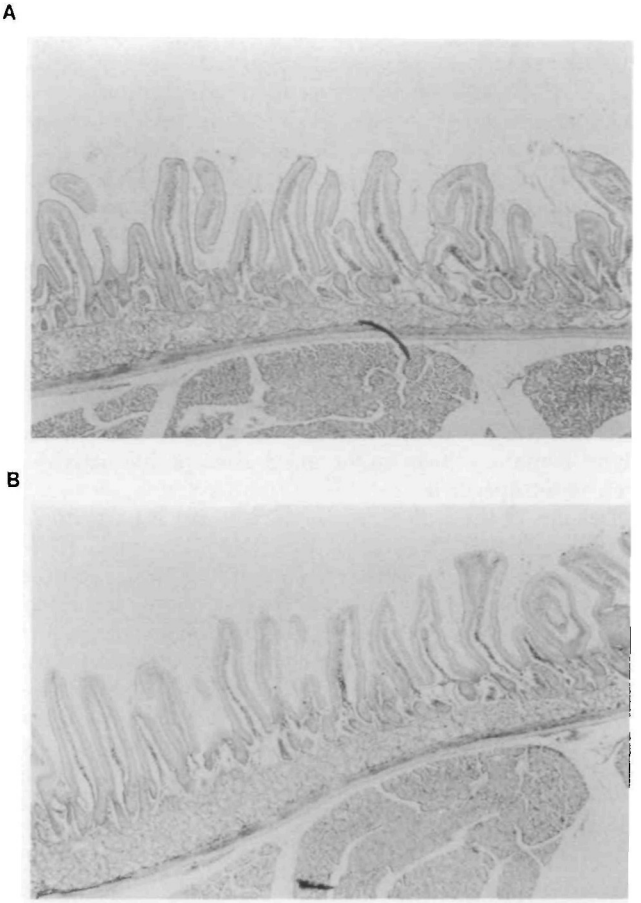

Fig. 5. **Immunohistochemical staining with the specific antibodies for the enzyme.** Immunohistochemical staining was performed using avidin DH and biotinylated horseradish peroxidase (Vector Lab.). A: With rabbit antibodies raised against the serine protease domain of the enzyme. Duodenal epithelia are weakly stained; intense staining is observed on the brushborder membranes. B: Without antibodies (control).

DISCUSSION

The cDNA cloned in this study codes for a membrane-bound serine protease. A comparison of partial sequences indicates this protease to be an ortholog of the membrane-bound arginine-specific serine protease that we previously purified from pig intestinal mucosa (*1*). Moreover, the rat enzyme was found to be highly homologous in domain structure and amino acid sequence with two similar enzymes recently found in different cells or tissues, mouse epithin [sequence identity, 94% (*5*)] and human matriptase/MT-SP1 [sequence identity, 81% (*6*, *7*)] (Fig. 1). Therefore, these enzymes are also thought to be the orthologs of the rat enzyme. It is noteworthy that in the sequences, KAIWVT (residues 743–748) and DSGGPLSS (residues 804–811) are completely identical among the rat, pig, mouse, and human enzymes. Ile745 in the former and Ser811 in the latter are mainly replaced by Cys in other trypsin/chymotrypsin-type serine proteases to form an additional disulfide bond. The three other disulfide bonds, as well as the catalytic Ser, His, and Asp residues in the catalytic domain, appear to be conserved among all trypsin/chymotrypsin-type serine proteases, including the present enzyme. Cys-731 is assumed to be disulfide-bonded to the non-catalytic domain.

The molecular mass of the rat enzyme detected by Western blot analysis of extracts of intestinal mucosa was about 70K, which is consistent with that (70K) of matriptase from human milk (*11*). Since the molecular size was not changed by reduction of the disulfide bonds, this 70 kDa protein is thought to be a single-chain inactive precursor. The membrane-bound, arginine-specific serine protease we purified from pig intestinal mucosa gave a band of about 50 kDa on SDS-PAGE under non-reducing conditions, and 32 kDa under reducing conditions (*1*). The N-terminal se-quence of the pig enzyme obtained by Edman degradation was X-Val-X-Gly-Glu (X: unidentified residue), which is homologous to the putative N-terminal sequences of the catalytic chains of the rat, mouse, and human enzymes (Fig. 1). The previously purified pig enzyme is supposed to be an activated two-chain form generated during purification by proteolysis, that lacks the N-terminal half of the non-catalytic domain including the transmembrane domain and the first of the two CUB domains.

In order to characterize the rat enzyme, a recombinant proform including the serine protease domain and the 19-residue segment of the activation peptide was expressed in *E. coli*. The active enzyme was obtained by *in vitro* refolding and subsequent autoprocessing of the purified proform, and purified by benzamidine affinity chromatography. Through these processes, proteins that failed to be correctly refolded and activated are thought to be removed. The specific activity toward Boc-Gln-Ala-Arg-MCA of the purified recombinant rat enzyme, which gave a single band on SDS-PAGE, however, was lower than that of the pig enzyme (*1*). One of the reasons for this difference may be inaccuracy in the quantification of the pig enzyme since the amount of purified enzyme was too small to determine with precision. In addition, the difference in the enzyme form used may be critical; the pig enzyme contains a large part of the non-catalytic domain, which might affect the specific activity as well as the specificity.

Despite of the difference in the specific activity toward Boc-Gln-Ala-Arg-MCA, the rat and pig enzymes showed similar substrate preferences (Table I and Fig. 2). Both enzymes cleaved several MCA substrates, mainly after arginine residues, and especially preferred to cleave the peptide bonds between paired basic residues, Arg-Arg or Arg-Lys, of certain neuropeptides. On the other hand, the substrate specificity of human MT-SP1, investigated using peptide libraries (*12*), appears to be somewhat different from the results obtained for the pig (*1*) and rat enzymes. The preferential cleavage between paired basic residues (Arg-Arg and Arg-Lys), observed for the pig and rat enzymes, is not remarkable for the human enzyme (*12*). Furthermore, the preferred cleavage sequences for human MT-SP1 were reported to be (Arg or Lys)-X-Ser-Arg-/-Ala and X-(Arg or Lys)-Ser-Arg-/-Ala, and Lys was also reported to be preferred as the P1 residue. On the other hand, dynorphin A and neurotensin were efficiently cleaved by the pig and rat enzymes at -Gly-Phe-Leu-Arg-/-Arg- and -Asn-Lys-Pro-Arg-/-Arg-, respectively, but not after Lys. Thus, their substrate specificities are apparently not in full accord with that of the human enzyme, although the preference for arginine at the P1 position is consistent among the three enzymes. The reason for this discrepancy is not clear at present, although it may be partly due to species differences.

The fairly limited substrate specificity and the major distribution in the intestine suggest some specific role(s) of this enzyme in this organ. Very recently, urokinase-type plasminogen activator (*11*, *12*) and hepatocyte growth factor (*11*) have been reported to be activated by human matriptase/MT-SP1, and a possible function of human matriptase/MT-SP1 in such events as tissue remodeling, cancer invasion and metastasis has been suggested. Although the human and mouse enzymes are also membrane-bound proteases present among others in the small intestine, their subcellular localization in the intestine has not been clarified. On the other hand, we demonstrate in the present study by immunostaining of the rat duodenal tissue that the enzyme is present on brushborder membranes of the intestine. Although this enzyme might be present in smaller amounts on the basolateral membranes or be secreted into the extracellular matrix, our findings suggest that it may participate in the processing or digestion of some specific proteins or peptides on the brushborder membranes. The possibility for its participation in the processing of some gastrointestinal polypeptides, however, remains to be examined. Further studies are thus necessary to elucidate the physiological role(s) of the enzyme.

REFERENCES

1. Tsuchiya, Y., Takahashi, T., Sakurai, Y., Iwamatsu, A., and Takahashi, K. (1994) Purification and characterization of a novel membrane-bound arginine-specific serine protease from porcine intestinal mucosa. *J. Biol. Chem.* **269**, 32985–32991
2. Matsushima, M., Ichinose, M., Yahagi, N., Kakei, N., Tsukada, S., Miki, K., Kurokawa, K., Tashiro, K., Shiokawa, K., Shinomiya, K., Umeyama, H., Inoue, H., Takahashi, T., and Takahashi, K. (1994) Structural characterization of porcine enteropeptidase. *J. Biol. Chem.* **269**, 19976–19982
3. Kitamoto, Y., Yuan, X., Wu, Q., McCourt, D.W., and Sadler, J.E. (1994) Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. *Proc. Natl. Acad. Sci. USA* **91**, 7588–7592
4. Yahagi, N., Ichinose, M., Matsushima, M., Matsubara, Y., Miki, K., Kurokawa, K., Fukamachi, H., Tashiro, K., Shiokawa, K., Kageyama, T., Takahashi, T., Inoue, H., and Takahashi, K. (1996) Complementary DNA cloning and sequencing of rat enteropeptidase and tissue distribution of its mRNA. *Biochem. Biophys. Res. Commun.* **219**, 806–812
5. Kim, M.G., Chen, C., Lyu, M.S., Cho, E.G., Park, D., Kozak, C., and Schwartz, R.H. (1999) Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics* **49**, 420–428
6. Lin, C.Y., Anders, J., Johnson, M., Sang, Q.A., and Dickson, R.B. (1999) Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J. Biol. Chem.* **274**, 18231–18236
7. Takeuchi, T., Shuman, M.A., and Craik, C.S. (1999) Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc. Natl. Acad. Sci. USA* **96**, 11054–11061
8. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by guanidium thiocyanate-PhOH-chloroform extraction. *Anal. Biochem.* **162**, 156–159
9. Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M.B. (1996) The I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression. *Genomics* **33**, 151–152
10. Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–88
11. Lee, S.-L., Dickson, R.B., and Lin, C.-Y. (2000) Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J. Biol. Chem.* **275**, 36720–36725
12. Takeuchi, T., Harris, J.L., Huang, W., Yan, K.W., Coughlin, S.R., and Craik, C.S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J. Biol. Chem.* **275**, 26333–26342
13. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680