Isolation and Characterization of a Novel Serine Proteinase Complexed with α2-Macroglobulin from Porcine Gastric Mucosa*

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Porcine stomach mucosa was found to contain a 740-kDa protein having endopeptidase activity toward peptide 4-methylcoumaryl-7-amide substrates and low molecular mass peptides. This protein was purified to an apparent homogeneity by a series of chromatographic steps on DEAE-cellulose, Sepharose CL-4B, hydroxylapatite, and fast protein liquid chromatography Mono Q columns. The protein was shown to be a complex of the plasma proteinase inhibitor α2-macroglobulin and a 25-kDa endopeptidase. The enzyme activity was completely inhibited by diisopropyl fluorophosphate, p-aminophenylmethanesulfonyl fluoride, leupeptin, antipain, bovine pancreatic trypsin inhibitor, soybean trypsin inhibitor, and ovomucoid, indicating that the entrapped enzyme is a serine proteinase. The proteinase could be released from α2-macroglobulin by mild acid treatment and the released enzyme showed activity toward protein substrates.

Substrate specificity studies using synthetic and peptide substrates indicated that the enzyme preferentially hydrolyzes Arg-X bonds and, to a much lesser extent, Lys-X bonds, and is apparently distinct from thrombin, kallikrein, plasmin, and other trypsin-like proteinases so far reported including trypsin. Thus, the present enzyme is thought to be a novel type of serine proteinase. The proteinase associated with α2-macroglobulin was also found in porcine intestinal mucosa, but not in plasma.

The stomach is a dilated segment of the digestive tract whose main function is to digest ingested food. The best studied stomach enzyme involved in the process of digestion in this organ is the aspartic proteinase pepsin (1-5). In contrast to this extracellular proteinase, very little is known of intracellular proteinases, although recently cathepsin E has been isolated and characterized (2, 6-9) and its structure analyzed (10-12). Mucosal extracts indeed contain a variety of peptide substrates and low molecular mass peptides that are thought to be involved in intracellular protein turnover (13-15). In addition, there must be some peptidases that function as proteolytic processing enzymes for certain biologically important proteins and their precursors since bioactive peptides such as gastrin, enkephalin, somatostatin, glucagon, and other peptides are known to be present in the stomach (16). However, none of the peptidases involved in the proteolytic processing of such peptides have yet been identified.

In an attempt to elucidate the functional role of proteolytic enzymes in the mammalian stomach, a high molecular weight protein that preferably hydrolyzes peptide 4-methylcoumaryl-7-amide (MCA) substrates at the Arg-MCA bond was found in the homogenate. In the present study, we purified the protein and characterized it in detail. The results reveal that the enzyme activity is due to an α2-macroglobulin-associated serine proteinase with a molecular weight of about 25,000. Further investigation of the enzymatic properties including substrate specificity and effects of various inhibitors indicated that this proteinase is distinct from any other known serine endopeptidases thus far described.

EXPERIMENTAL PROCEDURES AND RESULTS

Purification of a Protein with Boc-Gln-Arg-Arg-MCA Hydrolizing Activity from Porcine Stomach Mucosa—Table I summarizes the purification of a protein hydrolyzing Boc-Gln-Arg-Arg-MCA from porcine stomach mucosa. The detailed procedure is described in the Miniprint section. Anion-exchange column chromatography (DE-52 and Mono Q) always gave rise to low recovery of activity, but inclusion of these columns was eventually found to yield the protein in satisfactory purity as described in the following section. From 230 g of the mucosa, 1.4 mg of the protein was obtained with an overall yield of 2.2%.

Purity and Molecular Weight—Native PAGE using a gradient gel gave a single protein band associated with the activity (Fig. 5). Its apparent molecular mass was estimated to be approximately 730,000 by PAGE and 750,000 by gel filtration on Sepharose CL-6B (Fig. 6). Fig. 7 shows the results of SDS-PAGE of the sample in the presence and absence of 2-mercaptoethanol. A major protein band corresponding to M, = 360,000 and a very faint band corresponding to M, = 100,000 were observed under nonreducing conditions, while a major protein band (M, = 85,000) and other minor bands (M, = 80,000, 56,000, and 54,000) were separated under reducing conditions.

1 The abbreviations used are: MCA, 4-methylcoumaryl-7-amide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; TPCK, N'-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N'-tosyl-L-lysine chloromethyl ketone; BOC, N-butyloxycarbonyl; Z, benzoyloxycarbonyl; Bz, benzoyl; Suc, succinyl.

2 Portions of this paper (including "Experimental Procedures," part of "Results," Table 1, and Figs. 1-4, 6, 8-11, and 13-17) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
was separately applied to two well positions of a gradient PAGE gel (4–15%) without SDS. After electrophoresis at 4 °C, one lane was stained with Coomassie Brilliant Blue R-250 (lane 2), while the other was sliced into pieces of 3-mm width for overnight extraction in 0.5 ml of the routine assay buffer. Aliquots of the extracts were assayed for enzyme activity toward Boc-Gln-Arg-Arg-MCA and the relative activities are shown. Lane 1 shows the separation of molecular weight marker proteins (Pharmacia LKB Biotechnology Inc.: thyroglobulin (670 kDa), apoferritin (440 kDa), lactate dehydrogenase (140 kDa), and albumin (66 kDa)).

**FIG. 5. PAGE analysis of the purified protein.** The sample was separately applied to two well positions of a gradient PAGE gel (4–15%) without SDS. After electrophoresis at 4 °C, one lane was stained with Coomassie Brilliant Blue R-250 (lane 2), while the other was sliced into pieces of 3-mm width for overnight extraction in 0.5 ml of the routine assay buffer. Aliquots of the extracts were assayed for enzyme activity toward Boc-Gln-Arg-Arg-MCA and the relative activities are shown. Lane 1 shows the separation of molecular weight marker proteins (Pharmacia LKB Biotechnology Inc.: thyroglobulin (670 kDa), apoferritin (440 kDa), lactate dehydrogenase (140 kDa), and albumin (66 kDa)).

**FIG. 7. SDS-PAGE analysis of the purified protein.** A, the protein (2 µg) was electrophoresed in 7.5% PAGE gel in SDS under nonreducing (lane 1) and reducing (lane 2) conditions. The gels were stained with Coomassie Brilliant Blue. B, the protein (0.87 µg) was incubated with 8 µCi of [3H]DFP in 20 mM Tris-HCl (pH 7.5) and subjected to SDS-PAGE using 7.5% gel under reducing conditions, followed by fluorography. Molecular weight marker proteins used are: dimer of human α2-macroglobulin subunit (360 kDa), myosin (200 kDa), β-galactosidase (118 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa).

conditions. However, the sample treated with the serine proteinase inhibitor DFP (3H-labeled) produced only a single band with a molecular mass of approximately 28,000 on SDS-PAGE using 7.5% gel under reducing conditions, followed by fluorography. Molecular weight marker proteins used are: dimer of human α2-macroglobulin subunit (360 kDa), myosin (200 kDa), β-galactosidase (118 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa).

**Association of a Serine Protease with α2-Macroglobulin**—The results described above suggested that the purified protein is a complex of the plasma protein inhibitor α2-macroglobulin with a serine proteinase. To substantiate this point, several experiments were performed. The purified protein cross-reacted with goat anti-human α2-macroglobulin antibody (Fig. 8). Furthermore, direct electron microscopic observation of the protein revealed two similar molecular shapes (Fig. 9). One of them looks like the letter H, while the other is a rounded structure characterized by two thick units located roughly in parallel with small material between them. They are the characteristic shapes of α2-macroglobulin that underwent a drastic conformational change after reacting with proteinase (24).

**Release of a Serine Protease from the α2-Macroglobulin-proteinase Complex**—The results shown in Fig. 7 suggest that the proteinase is dissociable from the α2-macroglobulin-proteinase complex. It was found that exposure of the purified protein to pH 3 for 12 h did not cause significant change in the activity toward Boc-Gln-Arg-Arg-MCA substrate, but resulted in a remarkable increase in the activity toward protein substrates (Fig. 10). In addition, goat anti-human α2-macroglobulin antibody no longer precipitated the proteinase activity when the purified protein was previously treated at pH 3 (data not shown). These results clearly indicate that the proteinase is noncovalently associated with α2-macroglobulin, and is freed from steric restraint upon the acid treatment.

In order to obtain the pure enzyme from the acid-treated sample, several column chromatographic steps were tried under various conditions. The enzyme activity was completely lost on ion-exchange columns (Mono Q, Mono S, and DE-52). Poor recovery (about 20%) of the activity was also observed on the gel filtration over a Sephacryl S-300 column. Thus, none of these columns was found to be suitable for the purification. It should be noted that the gel filtration gave a single activity peak eluting at a position corresponding to M, = 25,000 (data not shown), a value close to that obtained in the [3H]DFP labeling experiment.

**Action on Synthetic Substrates**—No significant difference was observed when the activities toward a few MCA substrates were compared before and after the acid treatment of the protein. This was reasonable since the substrates are small enough to freely reach the active site irrespective of whether the proteinase is associated with the inhibitor. Thus, the activities on synthetic substrates were tested using the enzyme associated with α2-macroglobulin and the results are shown in Table II. The specificities of human thrombin (M, = 34,000), porcine tissue kallikrein (M, = 30,000), and porcine plasmin (M, = 80,000) are also included for comparison.

The enzyme was most active toward Boc-Gln-Gly-Arg-MCA. Boc-Gln-Arg-MCA, Boc-Gln-Arg-Arg-MCA, Boc-Phe-Ser-Arg-MCA, and Boc-Leu-Gly-Arg-MCA were hydrolyzed fairly well by the enzyme and to similar extents. A common feature of the substrates hydrolyzable by the enzyme was the presence of an Arg residue at the P1 position. Substrates containing Lys-MCA bonds were much less susceptible to the enzyme, and substrates for chymotrypsin were not cleaved at all. Little or no cleavage occurred when the MCA-derivatives of an amino acid or a dipeptide were tested. Thrombin very rapidly hydrolyzed the substrates Boc-Pro-Arg-MCA and Boc-Gln-Ala-Arg-MCA, while the tissue kallikrein most rapidly cleaved Boc-Gln-Ala-Arg-MCA, Boc-

**TABLE II Enzyme activities toward various MCA substrates**

| Substrate                          | Thrombin | Kallikrein | Plasmin |
|-----------------------------------|----------|------------|---------|
| Boc-Gln-Arg-Arg-MCA               | 100      | 100        | 100     |
| Boc-Gln-Gly-Arg-MCA               | 201      | 354        | 63      |
| Boc-Gln-Ala-Arg-MCA               | 121      | 3300       | 305     |
| Boc-Phe-Ser-Arg-MCA               | 105      | 142        | 97      |
| Boc-Leu-Gly-Arg-MCA               | 90       | 304        | 37      |
| Boc-Leu-Thr-Arg-MCA               | 48       | —          | —       |
| Boc-Gly-Arg-MCA                   | 42       | 11         | 13      |
| Boc-Val-Pro-Arg-MCA               | 41       | 4160       | 217     |
| Boc-Leu-Lys-MCA                   | 40       | 42         | 30      |
| Boc-Leu-Val-Lys-MCA               | 13       | 285        | 57      |
| Boc-Glu-Lys-MCA                   | 11       | —          | —       |
| Bz-Arg-MCA                        | 3        | —          | —       |
| Bz-Arg-MCA                        | 0.4      | —          | 71      |
| Suc-Ala-Ala-Pro-Phe-MCA            | 0        | —          | —       |
| Suc-Leu-Leu-Val-Tyr-MCA            | 0        | —          | —       |
| Arg-MCA                           | 0        | 5          | 0       |
| Leu-MCA                           | 0        | —          | —       |

*Not tested.*
Val-Leu-Lys-MCA, and Boc-Val-Pro-Arg-MCA. Plasmin cleaved Boc-Gln-Arg-MCA efficiently, but hydrolyzed Boc-Gln-Gly-Arg-MCA, a good substrate for the present enzyme, rather slowly. These results clearly show that the specificity of the current proteinase is different from those of thrombin, tissue kallikrein, and plasmin.

Action on Peptide Substrates—Four peptide substrates were tested for hydrolysis by the proteinase, and the results of high performance liquid chromatography analysis of the digests are shown in Fig. 11. As summarized in Fig. 12, cleavages occurred only on the COOH-terminal side of Arg or Lys residues. The specificity is thus consistent with that found with MCA substrates.

Effects of Inhibitors—The effects of various inhibitors on the activity were examined, and the results are shown in Table III. DFP, p-aminodiphenylmethanesulfonyl fluoride, antipain, leupeptin, and bovine pancreatic trypsin inhibitor inhibited strongly the activity of the \(\alpha_2\)-macroglobulin-associated proteinase. Strong inhibition by soybean trypsin inhibitor and ovomucoid was observed only after the purified protein was treated at pH 3, indicating again the release of the proteinase from \(\alpha_2\)-macroglobulin by this treatment. These results clearly indicate that the enzyme is a trypsin-like serine proteinase.

Presence of the \(\alpha_2\)-Macroglobulin-Proteinase Complex in Porcine Intestinal Mucosa—In order to examine whether or not the same \(\alpha_2\)-macroglobulin-proteinase complex is present in the mucosa of the porcine intestine, a partial purification experiment was conducted as described in the Miniprint section. Electrophoretic analysis of the fractions obtained by chromatography on Sepharose CL-6B revealed that \(\alpha_2\)-macroglobulin was eluted in fractions 45–50, which corresponded to one of the peaks with proteinase activity (Fig. 14). Association of the proteinase with \(\alpha_2\)-macroglobulin was confirmed by assaying the activity in the gel slice extracts as described under “Experimental Procedures.” The activities toward various MCA substrates were assayed for the proteinase in this peak. The relative activities were essentially the same as those of the purified stomach enzyme, suggesting that the same serine proteinase is present in the intestinal mucosa in the \(\alpha_2\)-macroglobulin-associated form. The specific enzyme activity toward Boc-Gln-Arg-MCA was estimated to be 23.9 milliunits/mg \(\alpha_2\)-macroglobulin (the amount of the inhibitor was densitometrically determined in PAGE of the above Sepharose CL-6B fraction). The value is about half the specific activity (42.1 milliunits/mg protein) of the \(\alpha_2\)-macroglobulin-protein complex purified from stomach.

As shown in Fig. 15, porcine plasma did not contain a significant amount of the inhibitor-proteinase complex.

**DISCUSSION**

In the present study we have isolated from porcine stomach mucosa a high molecular weight protein (approximately \(M_\text{r} = 740,000\)) with Boc-Gln-Arg-Arg-MCA hydrolyzing activity. Detailed characterization of the protein revealed that it is a complex of a major plasma protein, \(\alpha_2\)-macroglobulin, and an enzyme. This enzyme, which is responsible for the activity, was demonstrated to be a serine peptidase with a molecular mass of approximately 25,000 Da.

Cleavage specificity studies using synthetic and peptide substrates indicated that the enzyme preferentially hydrolyzes Arg-X bonds and, to a much lesser extent, Lys-X bonds. In addition, the enzyme appears to contain multiple amino acid side chain binding sites in the active site (25, 26). As suggested by the finding that tripeptide MCA substrates such as Boc-Gln-Arg-MCA and Boc-Gln-Gly-Arg-MCA, but not dipeptide MCA substrates such as Z-Arg-MCA and Z-Phe-Arg-MCA, are good substrates, filling at least four binding sites (\(S_1, S_2, S_3, \text{ and } S_4^\prime\)) seems to be a prerequisite for hydrolysis. Although further systematic kinetic studies are required to clarify the specificity of each binding site, the \(S_1^\prime\) subsite evidently favors basic (Arg, Lys) side chains. The enzyme has only endopeptidase activity, and its association with the plasma proteinase inhibitor \(\alpha_2\)-macroglobulin is consistent with the well-known fact that the inhibitor interacts only with enzymes having endopeptidase activity (27).

The mechanism of inhibition of proteinase activity by \(\alpha_2\)-macroglobulin is thought to be physical trapping of proteinases by the inhibitor (27, 28). We suspected that the enzyme in the present study might be one of the well-characterized proteinases. Several authors have reported that isolated \(\alpha_2\)-macroglobulin fractions have low kallikrein-like (29, 30) and trypsin-like (31) activities. More recently, thrombin associated with \(\alpha_2\)-macroglobulin was isolated from mammalian cells cultured in the presence of the small proteinase inhibitor leupeptin (32, 33). In this context, it is particularly interesting to compare the current proteinase with kallikrein (34) and thrombin (35) since these enzymes appear to have overlapping enzymatic properties such as molecular size (\(M_\text{r} = 25,000–35,000\)) and endopeptidase nature with trypsin-like cleavage specificity. However, the results shown in Table II indicate that the \(\alpha_2\)-macroglobulin-associated enzyme has a substrate...
specificity clearly distinct from those of tissue kallikrein and thrymbin.

It should be noted that the substrate specificity of the enzyme is somewhat similar to that of rat lung trypsinase; the relative activities of the trypsin toward Boc-Phe-Ser-Arg-MCA, Boc-Val-Pro-Arg-MCA, and Boc-Val-Leu-Lys-MCA are 100, 50.6, and 6.1 (36), respectively, while those of the present enzyme are 100, 39, and 12.9, respectively. In Table II.

In contrast, the behavior of the two enzymes toward polypeptide proteinase inhibitors such as bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor is different. The proteinase dissociated from α2-macroglobulin has been completely inhibited by the above inhibitors, but the trypsinase was reported to be inhibited by a limited extent even at a higher inhibitor concentration (36). Furthermore, native trypsinase is known to be a tetramer (Mₛ = 144,000) consisting of two species of subunits (Mₛ = 30,900 and 31,600) (37, 38). Smith et al. (37) reported the failure of pure α₂-macroglobulin to inhibit human lung trypsinase, suggesting that this enzyme may be too large to be trapped by α₂-macroglobulin (28). Additional differences between the two enzymes are also observed in pH profiles for stability and activity. From these considerations, the enzyme found in the current study is thought to be α₂-macroglobulin-trypsin complex. Furthermore, we could not find the corresponding free proteinase in the homogenate.

We have considered the possibility that the α₂-macroglobulin-proteinase complex is artificially produced during purification. Since methylamine treatment is known to rapidly inactivate α₂-macroglobulin (39), addition of the reagent could prevent the proteinase trapping by inhibitor if the enzyme is originally present in a free form in the tissue homogenate. However, purification experiments with or without methylamine produced no significant difference in the yield of the α₂-macroglobulin-proteinase complex. Furthermore, we could not find the corresponding free proteinase in the homogenate of the stomach mucosa and the yield of the α₂-macroglobulin-proteinase complex did not change significantly whether it was purified from fresh mucosa immediately after killing or after storage of the mucosa at 20 °C for months. Moreover, inclusion of trypsin in the homogenization buffer neither resulted in recovery of the α₂-macroglobulin-trypsin complex nor affected the activity in fractions containing the α₂-macroglobulin complex of the present enzyme. These results strongly suggest that the proteinase-α₂-macroglobulin complex does not form during storage or after homogenization and that the enzyme is only present in a form associated with α₂-macroglobulin in vivo. The same complex is also present in the intestinal mucosa, but not in the plasma.

At present, the physiological significance of the complex in the digestive organs is not clear. The enzyme is very stable when complexed with α₂-macroglobulin, but appears to be rendered unstable after release from α₂-macroglobulin. Therefore, α₂-macroglobulin may be important in stabilizing the enzyme and/or restricting the enzyme action only to low molecular weight peptides. To clarify its physiological roles, further studies, including detailed characterization of the trapped enzyme, identification of cells producing the enzyme, and localization of the α₂-macroglobulin-proteinase complex in the tissues, are necessary. However, the present finding that the proteinase associated with α₂-macroglobulin hydrolyzes peptide substrates at the Arg-X and Lys-X bonds tempts us to speculate its possible involvement in the proteolytic processing or degradation of some bioactive peptides known to be present in the gastrointestinal tract. Such a possibility is now under examination.

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References

1. Frutos, J. J. (1971) The Enzymes 3, 119-184.

2. Santell, I. M. (1960) Gastroenterology 37, 659-669.

3. Kageyama, T., and Takahashi, K. (1983) J. Biochem. (Tokyo) 93, 743-754.

4. Sogawa, K., Fujii-Kuriyama, Y., Minakami, Y., Ichihara, Y., and Takahashi, K. (1984) J. Biol. Chem. 259, 5306-5311.

5. Hayano, T., Sogawa, K., Ichihara, Y., Fujii-Kuriyama, Y., and Takahashi, K. (1988) J. Biochem. (Tokyo) 97, 725-735.

6. Matsuzaki, O., and Takahashi, K. (1988) Biomed. Res. 9, 559-565.

7. Sasaki, T., T. Taggart, R. T., Shiraishi, T., Brench, T., Reiz, W. A., Heath, R. W., Valler, M. J., and Kay, J. (1987) Gastroenterology 93, 77-84.

8. Athauda, S. B. P., Takahashi, T., Inoue, H., Ichinose, M., and Takahashi, K. (1991) FEMS Lett. 292, 53-56.

9. Azuma, T., Pala, G., Mohanlal, T. K., Courvireur, J. M., and Taggart, R. T. (1989) J. Biol. Chem. 264, 16744-16753.

10. Athauda, S. B. P., Takahashi, T., Kageyama, T., and Takahashi, K. (1990) Biochem. Biophys. Res. Commun. 164, 785-788.

11. Athauda, S. B. P., Takahashi, T., Kageyama, T., and Takahashi, K. (1991) Biochem. Biophys. Res. Commun. 175, 152-158.

12. Wooley, D. E., Zucker, J. S., Green, G., and Evanson, J. M. (1976) Biochem. J. 153, 119-126.

13. de Brum, P. A. F., Giffrein, G., and Verspaget, H. W. (1987) Cancer Res. 47, 4654-4657.

14. Barrett, A. J. (1977) in Proteins in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 209-248, North-Holland Publishing Co., Amsterdam.

15. Fujita, T., Kanno, T., and Kobayashi, S. (1988) in The Paraneuron, pp. 165-184, Springer-Verlag, Tokyo.

16. Barrett, A. J. (1980) Biochem. J. 187, 909-912.

17. Barrett, A. J., and Kirchke, H. (1981) Methods Enzymol. 80, 553-561.

18. Sogawa, K., and Takahashi, K. (1975) J. Biochem. (Tokyo) 84, 763-770.

19. Laemmli, U. K. (1970) Nature 227, 680-685.

20. Smith, J. K., Kroko, L. E. Hernanzon, G., Mollis, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. A., Coote, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76-85.

21. Tockman, H., Stachelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.

22. Osada, T., Sasaki, T., and Ikai, A. (1986) J. Biochem. (Tokyo) 100, 212-214.

23. Nishigai, M., Osada, T., and Ikai, A. (1985) Biochim. Biophys. Acta 831, 236-241.

24. Schechter, I., and Berger, A. (1997) Biochem. Biophys. Res. Commun. 27, 317-326.

25. Starkey, M. W., and Johnson, D. A., and Chretien, G. (1976) Naunish-Schmiderberg's Arch. Pharmac. 294, 76-84.

26. McConnell, D. J. (1972) Clin. Invest. 51, 1611-1623.

27. Laurell, C. B., and Jeppson, J.-O. (1975) in The Plasma Proteins (Putnam, F. W., ed) pp. 229-254, Academic Press, New York.

28. Tsuchi, A., and Kuroki, K. (1989) J. Biol. Chem. 264, 16093-16099.

29. Tsuchi, A., Araki, T., Fucinetti, P. S., Langmore, J. P., and Kurachi, K. (1991) Biochem. Biophys. Acta 103, 85-92.

30. Fiedler, P., Fink, E., Tschesc, H., and Fritz, H. (1981) Methods Enzymol. 80, 493-522.

31. Lundblad, B. L., Kingdon, H. S., and Mann, G. M. (1976) Methods Enzymol. 45, 156-176.

32. Kido, H., Fukuson, N., and Katsumama, N. (1986) Arch. Biochem. Biophys. 238, 430-443.

33. Smith, T. J., Houghton, M. W., and Johnson, D. A. (1984) J. Biol. Chem. 259, 10461-10465.

34. Cruenlsh, J. A., Seidah, N. G., Marcinkiewicz, M., Hameil, J., Johnson, D., and Chretien, M. (1987) J. Biol. Chem. 262, 1363-1373.

35. Travis, J., and Salvesen, G. S. (1985) Annu. Rev. Biochem. 52, 655-709.

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Experimental Procedures

**Extracellular Activity**

The enzyme activities toward porcine MCA substrates were assayed according to the method of Ballinger et al. (12) using the MCA assay. The reaction was initiated by the addition of 100 μl of 10 μM MCA substrate solution to 100 μl of enzyme solution in 0.1 M sodium acetate buffer, pH 6.0, and the reaction was incubated at 37 °C for 1 h. The absorbance was measured at 405 nm in a spectrophotometer. The activity was expressed as the amount of enzyme that released 1 μmol of p-nitroanilide from the substrate per minute.

**Digestion of Peptide Substrates and Analysis of the Digests**

The purified protein (200 μl) was incubated with pepsin (3 mg/ml, pH 1.5) in 50 mM Tris-HCl buffer, pH 7.5, for 3 h. The reaction was stopped by the addition of 1 M HCl to a final concentration of 0.5 M. The digest was then applied to a Sephadex G-25 column equilibrated with 0.2 M NaCl, pH 4.5, and the effluent was monitored at 280 nm.

**Immunoprecipitation**

Equal volumes of the purified enzyme were mixed with various amounts of anti-serine proteinase monoclonal antibodies and incubated at 4 °C for 1 h. The reaction was then centrifuged, and the supernatant was subjected to SDS-PAGE. The protein bands were visualized by Coomassie Brilliant Blue staining.

**Electron Microscopy**

Enzyme activity was determined using a Hitachi H7000 electron microscope. Thin sections of the purified protein were stained with uranyl acetate and lead citrate and examined at 80 kV.

**Partial Purification of α2-Macroglobulin from Porcine Plasma**

The α2-macroglobulin was isolated from porcine plasma by density gradient centrifugation and ultracentrifugation. The α2-macroglobulin was then purified by ion exchange chromatography on a DEAE-cellulose column. The protein was eluted with a linear gradient of 0.1 M NaCl in 0.01 M Tris-HCl, pH 7.5, and the fractions containing the protein were pooled and dialyzed against 0.1 M Tris-HCl, pH 7.5.

**Isolation and Characterization of a Novel Serine Proteinase Complexed with α2-Macroglobulin from Porcine Gastric Mucosa**

A novel serine proteinase was isolated from porcine gastric mucosa by affinity chromatography on a DEAE-cellulose column. The protein was eluted with a linear gradient of 0.1 M NaCl in 0.01 M Tris-HCl, pH 7.5, and the fractions containing the protein were pooled and dialyzed against 0.1 M Tris-HCl, pH 7.5.

**Partial Purification of α2-Macroglobulin from Porcine Plasma**

The α2-macroglobulin was purified by ion exchange chromatography on a DEAE-cellulose column. The protein was eluted with a linear gradient of 0.1 M NaCl in 0.01 M Tris-HCl, pH 7.5, and the fractions containing the protein were pooled and dialyzed against 0.1 M Tris-HCl, pH 7.5.

**Hydrolytic Properties**

The protein was incubated at 37 °C with 0.1 M sodium phosphate buffer, pH 7.5, and the reaction was stopped by the addition of 4 M guanidine hydrochloride. The enzyme activity was then measured using the MCA assay.
**α₂-Macroglobulin-complexed Serine Proteinase**

**Fig. 4.** HPLC on a Mono Q column. The sample containing 3 mg of protein was loaded on a Mono Q column. The column was eluted with a linear concentration gradient of CH₃COONa/H₂O (0-0.5 M) at a flow rate of 0.5 ml/min, and fractions of 0.5 ml were collected.

**Fig. 5.** Electron micrograph of the purified protein. A: Protein was negatively stained with 5 % uranyl acetate without prior fixation. The scale bar represents 50 nm. B: Two different molecular shapes of the protein are schematically shown based on the electron microscopic observation.

**Fig. 6.** Molecular weight estimation of the protein by Sephacryl CL-6B gel filtration. The protein was applied to a column (2.7 x 93 cm) of Sephacryl CL-6B and eluted with 0.05 M potassium phosphate buffer (pH 6.7) containing 0.1 M NaCl at a flow rate of 50 ml/h. The elution position of the protein is indicated by an arrow. Trypsinogen (20,700 kDa), apetin (AF, 240 kDa), and cathepsin (Cat, 158 kDa) were used as molecular weight marker proteins.

**Fig. 7.** Reaction of the purified protein with goat anti-human α₂-macroglobulin antiserum. A: Lane 1 shows native PAGE analysis of the protein (0.78 µg) using a gradient gel (2-15%). Lane 2 is the Western blot analysis. B: Five micrograms of the protein (1.0 µg) was mixed with diluted amounts of the antiserum (2.5, 5.0, 10.0, and 25.0 µl). Immunoconjugates were removed by adsorption with 0.5 M ammonium acetate (pH 8.0) for 2 min and assayed for its protein-ase activity. The enzyme activity was immunoprecipitated with the anti-human α₂-macroglobulin antiserum.

**Fig. 8.** Enzyme activity toward protein substrates. The purified protein fraction was dialyzed against 0.1 M phosphate buffer (pH 7.0) at 4 °C for 2 h, and then against 0.5 M potassium phosphate (pH 7.5). The enzymatic activities of the treated (open columns) and untreated (hatched columns) samples were assayed by the fluorescence method using the bio-degraded casein (a), azocasein (b), and bovine serum albumin (c). The activities are expressed relative to the casein-hydrolysing activity of the untreated protein samples.

**Fig. 9.** HPLC profiles of digestion mixtures of peptides by the protease α₂-Macroglobulin (α₂-MG), BSA (B), and Bovine (B) VIPI) and standards (s) digested at 37 °C for 3 h with the α₂-macroglobulin-associated enzyme were analyzed by HPLC. All the peaks were collected and analyzed for the composition (a) 1: RPTL; 2: YQFPRL; 3: original peptide; 4: YQFPRL; 5: BQFPAR; 6: YQFPAR; 7: GDPGR; 8: DQGRPR; 9: original peptide; 10: HSDYAVTTNYT; 11: HSDYAVTTNYT; 12: HSDYAVTTNYT; 13: HRQGQKNSKTVIVLTH; 14: original peptide; 15: HPEIPMHR; 16: RPTL; 17: original peptide.
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Figure 13: DEAE-cellulose chromatography of an intestinal mucosal extract. The supernatant of the crude extract of porcine intestinal mucosa (15 g) was applied to a column (2.1 × 20 cm) of DE-52 and the absorbed proteins were eluted with a linear concentration gradient of NaCl using 1:1 ratio of 20 mM potassium phosphate (pH 7.4) and the same buffer containing 0.3 M NaCl. Flow rate was 48 ml/h and fractions of 11.4 ml were collected. The fractions were assayed for the enzyme activity with the substrate Boc-Glu-Arg-Arg-MCA.

Figure 14: Sepharose CL-4B chromatography. The sample derived from the previous DEAE-cellulose column was concentrated by precipitating with (NH₄)₂SO₄ at 90% saturation and applied to a Sepharose CL-4B column (3.7 × 93 cm). The proteins were eluted with 50 mM potassium phosphate (pH 6.7) containing 0.2 M NaCl at a flow rate of 30 ml/h. Fractions of 11 ml were collected and assayed for the enzyme activity. Electrophoretic analysis using a native, polyacrylamide gel showed that a protein corresponding to α₂-macroglobulin was eluted in the fractions between 48 and 54.

Figure 15: Sepharose CL-4B chromatography of porcine plasma protein. The plasma protein precipitated with PEG 6000 at 5.5-12.5% (v/v) was dissolved in a small volume of 20 mM sodium phosphate (pH 7.0) containing 0.15 M NaCl. The sample (11 mg protein) was applied to a Sepharose CL-4B column (5.7 × 90 cm) and eluted with 50 mM potassium phosphate (pH 6.7) containing 0.2 M NaCl in a flow rate of 30 ml/h. Fractions of 11 ml were collected and assayed for enzyme activity toward Boc-Glu-Arg-Arg-MCA. Elution positions of α₂-macroglobulin were also determined by electrophoretic analysis. A: Elution profile of the proteins. B: Electrophoretic analysis for α₂-macroglobulin. Lane 1, immunoor-α₂-macroglobulin; lane 2, fraction 44; lane 3, fraction 47; lane 4, fraction 50; lane 5, fraction 52; lane 6, fraction 54; fraction 56. The DISK gel (200 kDa) and α₂-macroglobulin (140 kDa) were used as molecular weight marker proteins. Note the enzyme activity is absent in the fractions 44-47 that contains α₂-macroglobulin.

Figure 16: pH Dependence of the protease activity. The buffers were used at a final concentration of 50 mM potassium phosphate buffer (pH 7.0) and 50 mM borate-acetate buffer (pH 8.0). A and B show the relative activity (%).

Table 1: Purification of a protein with Boc-Glu-Arg-Arg-MCA hydrolyzing activity from porcine stomach mucosa

| Step                  | Protein Activity | Specific Activity | Purification | Yield |
|-----------------------|------------------|-------------------|--------------|-------|
| Crude extract         | 1080             | 2728              | 0.35         | 1     | 100  |
| DE 52                 | 410              | 465               | 1.13         | 3.2   | 17   |
| Sepharose CL-4B       | 23               | 363               | 15.8         | 45.1  | 13   |
| Hydrosylate           | 9.0              | 248               | 27.6         | 78.9  | 9.1  |
| Mono Q                | 1.4              | 39                | 42.1         | 120   | 2.2  |