Journal of Enzyme Inhibition and Medicinal Chemistry, June 2003 Vol. 18 (3), pp. 219–224

Taylor & Francis
healthsciences

Inhibition of Human Pepsin and Gastricsin by α_2-Macroglobulin

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(Received 29 October 2002)

The inhibitory effects of human α_2-macroglobulin (α_2-M), a major plasma proteinase inhibitor, on human pepsin and gastricsin were investigated. The activities of pepsin and gastricsin towards a protein substrate (reduced and carboxymethylated ribonuclease A) were significantly inhibited by α_2-M at pH 5.5, whereas those towards a peptide substrate (oxidized insulin B-chain) were scarcely inhibited. Under these conditions at pH 5.5, pepsin and gastricsin cleaved α_2-M mainly at the His^{694}–Ala^{695} bond and Leu^{697}–Val^{698} bond, respectively, in the bait regions sequence of α_2-M. The conformation of α_2-M was also shown to be markedly altered upon inhibition of these enzymes as examined by native polyacrylamide gel electrophoresis and electron microscopy. These results show the entrapment and concomitant inhibition of those proteinases by α_2-M.

Keywords: α_2-Macroglobulin; Aspartic proteinase; Pepsin; Gastricsin

Abbreviations: α_2-M, α_2-macroglobulin; M_r, relative molecular mass; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography

INTRODUCTION

Human α_2-macroglobulin (α_2-M) is a plasma glycoprotein ($M_r \sim 725$ K) known to inhibit various kinds of proteinases by a molecular trapping mechanism.[1,2] It is composed of four identical subunits of $M_r \sim 185$ K,[3,4] which are linked in pairs by disulfide bonds, the two pairs being associated non-covalently to form the native, tetrameric molecule. Inhibition of proteinases by α_2-M is thought to occur generally through complex formation initiated by limited proteolysis in the bait region of α_2-M with a concomitant conformational change and entrapment of the proteinases.[3,4] Previously, we investigated the inhibition of human cathepsin E and immunodeficiency virus (HIV) proteinase by α_2-M.[5,6] Cathepsin E is a dimeric aspartic proteinase ($M_r \sim 84$ K) with two active sites per molecule, whereas HIV proteinase is a dimeric aspartic proteinase ($M_r \sim 22$ K) with a single active site per molecule. The activity of cathepsin E toward a protein substrate (reduced and carboxymethylated (RCm-)ribonuclease A) was completely inhibited by α_2-M[5], whereas that of HIV proteinase toward the same substrate was scarcely inhibited by α_2-M (Athauda and Takahashi, unpublished data). Therefore, it is interesting to investigate whether and in what manner α_2-M inhibits the activity of monomeric aspartic proteinases with intermediate molecular sizes. So far, however, few studies have been performed on the effects of α_2-M on this type of aspartic proteinase. In the present study, we have investigated the effects of α_2-M on human pepsin (also called pepsin A) and gastricsin (also called pepsin C), which are typical monomeric aspartic proteinases ($M_r \sim 37$ K) with a single active site per molecule. Pepsin and gastricsin are major gastric proteinases optimally active near pH 2 and 3, respectively. However, they have low but significant activity at pH 6.0–6.5 towards oxidized insulin B-chain[7] and casein (Athauda and Takahashi, unpublished data), where α_2-M is fairly stable against

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/1475636031000101246

acid denaturation. Pepsinogen and progastricsin are also known to be present in serum and some other biological fluids and tissues,[8,9] where they are potentially active, and α_2-M is present not only in serum but also in certain tissues.[10] Therefore, if these zymogens are activated by some means, they might be inhibited by α_2-M. The results of the present study show that both enzymes are trapped and significantly inhibited by α_2-M through limited proteolysis at unique peptide bonds in the bait region with concomitant conformational changes in α_2-M.

MATERIALS AND METHODS

Materials

Human pepsin (pepsin A-5, the major component of human pepsin isozymes) and gastricsin (pepsin C) were purified to homogeneity as previously described.[11] The B-chain of oxidized bovine insulin and bovine pancreatic ribonuclease A were purchased from Sigma and Worthington, respectively. Reduced and carboxymethylated (RCm-)ribonuclease A was prepared according to Crestfield *et al.*[12] α_2-M was purified from pooled human blood samples as previously described.[13]

Treatment of Proteinases with α_2-M

Each proteinase (final concentration, 125 nM) was treated with α_2-M at an enzyme: α_2-M molar ratio of 2:1 in 0.25 M sodium acetate buffer, pH 5.5, or in 0.25 M sodium phosphate buffer, pH 6.0, 6.5, 7.0 or 7.4, at 37°C for 30 min unless otherwise specified. Trypsin (used as a reference protein) was treated under the same conditions in 0.25 M sodium phosphate buffer, pH 6.5.

Digestion of Protein and Peptide Substrates and Analysis of the Digests

RCm-ribonuclease A (30 nmol) was digested separately with 0.3 nmol each of pepsin and gastricsin and of α_2-M-treated pepsin and gastricsin in 300 μl of 0.5 M sodium acetate buffer, pH 5.5 at 37°C for 2 h. The B-chain of oxidized insulin (100 nmol) was also digested under the same conditions with 0.02 nmol of each enzyme in 370 μl of the same buffer, pH 5.5. Aliquots of 150 μl were removed at appropriate intervals and subjected to HPLC using a Hitachi 655A-11 system on a column (0.46 × 25 cm) of TSKgel ODS-120T (Tosoh Co.). Elution was performed with a gradient of acetonitrile (0–60% in 60 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min and monitored at 215 nm.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed according to Laemmli.[14] About 5 μg each of proteinase-treated α_2-M and native α_2-M samples were separately mixed with the SDS-PAGE sample buffer containing 2-mecrcaptoethanol and subjected to PAGE in 7.5% acrylamide-0.1% methylene bisacrylamide gel in the presence of 0.1% SDS. PAGE under non-denaturing conditions was performed as follows: about 2 μg each of proteinase-treated α_2-M and native α_2-M were subjected to electrophoresis on a phase gradient gel (4–15%) under non-denaturing conditions in the Phast System (Pharmacia).

Analysis of the Sites of Cleavage in α_2-M by Proteinases

About 0.5 nmol of proteinase-treated α_2-M was subjected to SDS-PAGE as described above. Polypeptides in the gel were electro-blotted onto polyvinylidene difluoride membrane with 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid buffer, pH 11, and the membrane was stained with Coomassie Brilliant Blue R-250. Polypeptide bands were cut out, washed with distilled water and subjected to amino acid sequence analysis in an Applied Biosystems pulse-liquid protein sequencer (477A/120A).

Electron Microscopy

Electron micrographs of α_2-M treated with each proteinase at different pH values as described above were taken with a Hitachi H-7000 electron microscope. Prior to application of a sample to a grid with glow-discharged carbon-coated Formvar film, the protein concentration of the sample solution was adjusted to approximately 20 μg/ml. The excess solution on the grid was removed by blotting, and the sample was negatively stained with 3% uranyl acetate, pH 4.5.

RESULTS

Inhibition of the Proteinases by α_2-M

When RCm-ribonuclease A was incubated with pepsin at a molar ratio of 100:1 at pH 5.5 for 2 h, about 95% of the substrate protein disappeared and several peptide peaks appeared on HPLC (Fig. 1a). However, when the protein was incubated under the same conditions with a pepsin sample pretreated at pH 5.5 with α_2-M (molar ratio, pepsin: α_2-M = 2:1), about 30% of the substrate protein remained and a fewer peptide peaks appeared in smaller amounts

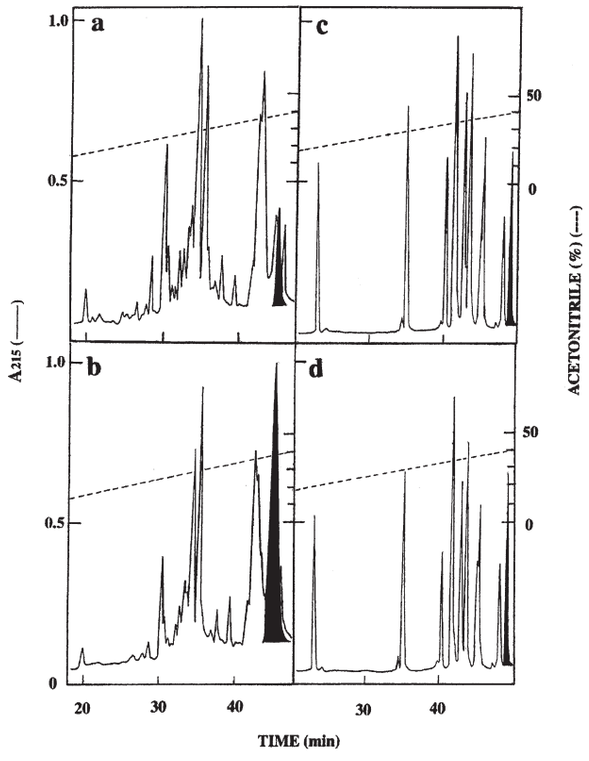

FIGURE 1 HPLC patterns of 2 h pepsin digests at pH 5.5 of RCm-ribonuclease A and oxidized insulin B-chain on a TSKgel ODS-120T column. (a) RCm-ribonuclease A + enzyme, (b) RCm-ribonuclease A + α_2-M-treated enzyme, (c) oxidized insulin B-chain + enzyme, (d) oxidized insulin B-chain + α_2-M-treated enzyme. The peak of the substrate remaining uncleaved is shaded.

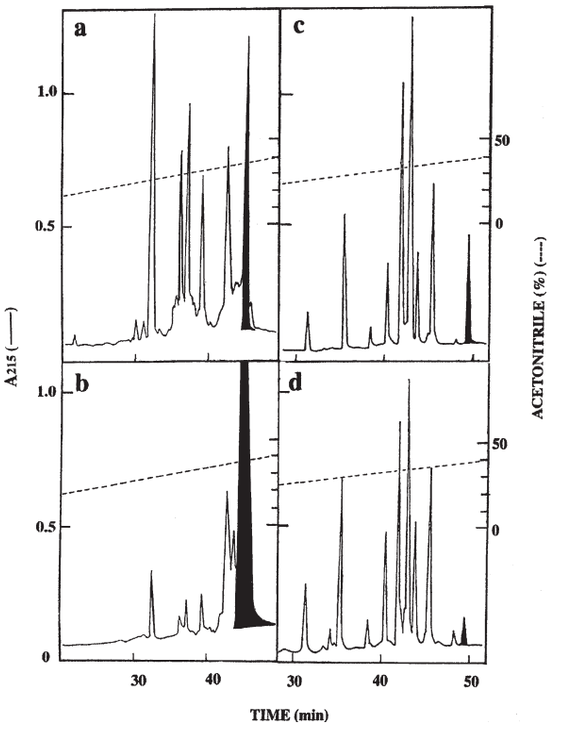

FIGURE 2 HPLC patterns of 2 h gastricsin digests at pH 5.5 of RCm-ribonuclease A and oxidized insulin B-chain on a TSKgel ODS-120T column. (a) RCm-ribonuclease A + enzyme, (b) RCm-ribonuclease A + α_2-M-treated enzyme, (c) oxidized insulin B-chain + enzyme, (d) oxidized insulin B-chain + α_2-M-treated enzyme. The peak of the substrate remaining uncleaved is shaded.

(Fig. 1b). Experiments were also performed at various molar ratios of pepsin and α_2-M (0.5:1, 1:1, 1.5:1, 3:1 and 4:1) at pH 5.5 (data not shown). Similar inhibition was observed at molar ratios of 0.5:1 and 1:1 as at 2:1, whereas, the inhibition was less remarkable at molar ratios of 3:1 and 4:1. These results suggest the binding stoichimetry of pepsin:α_2-M to be 2:1 (mol/mol). On the other hand, HPLC patterns obtained for 2 h digests of oxidized insulin B-chain by pepsin and by α_2-M-treated pepsin were very similar (Fig. 1c and d).

When RCm-ribonuclease A was incubated with gastricsin under the same conditions as above, about 80% of the substrate protein disappeared and several peptide peaks appeared on HPLC (Fig. 2a). However, when the protein was incubated under the same conditions with a gastricsin sample pretreated at pH 5.5 with α_2-M (molar ratio, gastricsin: α_2-M = 2:1), about 80% of the substrate protein remained and fewer peptide peaks appeared in much smaller amounts (Fig. 2b). Similar experiments were also performed at various molar ratios of gastricsin and α_2-M (1:1, 1.5:1 and 4:1) at pH 5.5 (data not shown). At molar ratios of 1:1 and 1.5:1, the activity was strongly inhibited as at 2:1, whereas, at a molar ratio of 4:1, the activity was only partially inhibited. These results suggest the binding stoichimetry of gastricsin: α_2-M to be 2:1 (mol/mol). On the other hand, HPLC patterns obtained for 2 h digests of oxidized insulin B-chain by gastricsin and by α_2-M-treated gastricsin were very similar, though not identical (Fig. 2c and d).

Limited Proteolysis and the Cleavage Sites in α_2-M

Figure 3a shows the SDS-PAGE patterns under reducing conditions of native α_2-M and α_2-M samples treated with trypsin at pH 6.5 and with pepsin at pH 5.5. Similar to the trypsin-treated α_2-M, the pepsin-treated α_2-M gave two major bands (I and II) at the position of M_r near 97 K, whereas native α_2-M gave one band at the position of $M_r \sim 185$ K. These results indicate that the above two major peptides were produced by limited proteolysis of the bait region of α_2-M by pepsin as in the cases of trypsin and other proteinases. Nearly complete cleavage was observed at pH 5.5 at a pepsin: α_2-M molar ratio of 2:1. The extent of cleavage decreased at higher pH; complete cleavage occurred in 30 min at a pepsin: α_2-M molar ratio of 8:1 (data not shown).

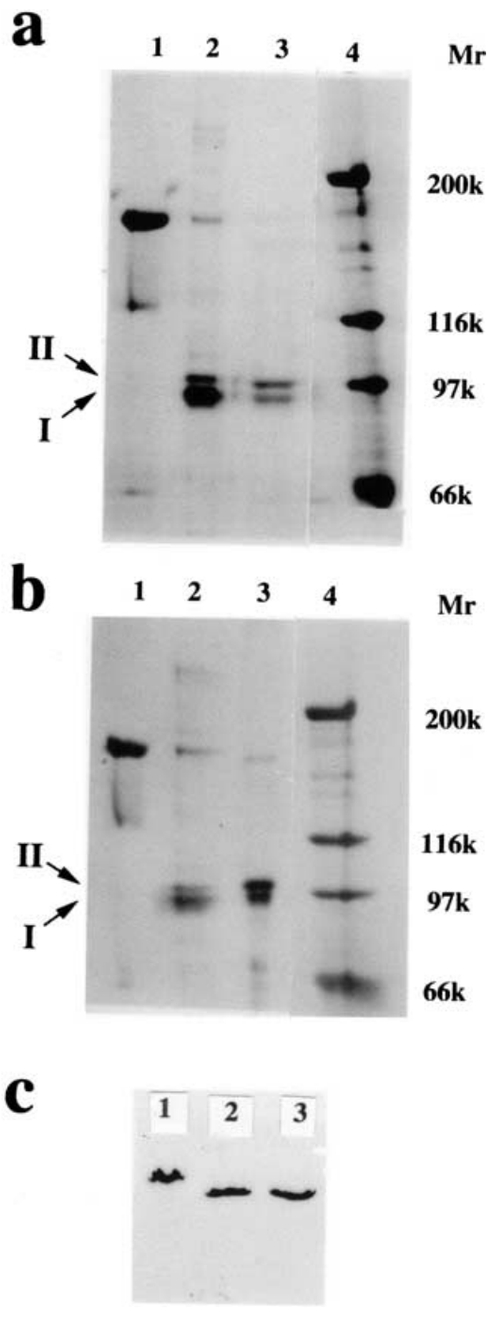

FIGURE 3 PAGE of intact and proteinase-treated α_2-M. (a) SDS-PAGE under reducing conditions of pepsin-treated α_2-M. Lane 1, α_2-M; lane 2, α_2-M treated with trypsin at pH 6.5; lane 3, α_2-M treated with pepsin at pH 5.5; and lane 4, molecular weight marker proteins. (b) SDS-PAGE under reducing conditions of gastricsin-treated α_2-M. Lane 1, α_2-M; lane 2, α_2-M treated with trypsin at pH 6.5; lane 3, α_2-M treated with gastricsin at pH 5.5; and lane 4, molecular weight marker proteins. (c) Native PAGE. Lane 1, α_2-M; lane 2, α_2-M treated with pepsin at pH 5.5; lane 3, α_2-M treated with gastricsin at pH 5.5.

Figure 3b shows similar SDS-PAGE patterns obtained with gastricsin. Gastricsin also gave two major polypeptide bands (I and II) at the position of M_r near 97 K. At a gastricsin: α_2-M molar ratio of 2:1, the bait region of α_2-M appeared to be largely cleaved at pH 5.5. The cleavage was incomplete at higher pH; complete cleavage at higher pH was observed at a gastricsin: α_2-M molar ratio of 16:1 in 30 min (data not shown).

The N-terminal amino acid sequences of the band I and II peptides produced by pepsin at pH 5.5 were determined as follows.

Band I :

Ser–Val–Ser–Gly–Lys–Pro–Gln–Tyr––––

Band II Major :

Val–His–Val–Glu–Glu–Pro–His–Thr––––

Band II Minor :

Tyr–Glu–Ser–Asp–Val–Met–Gly–Arg––––

The band I peptide had the N-terminal sequence identical with that of native α_2-M.[15] The band II peptide was a mixture of one major and one minor peptide (molar ratio, 6:1), with a sequence starting from Val^{698} and that from Tyr^{685}, respectively. Thus, pepsin cleaved the bait region of α_2-M at the two sites at pH 5.5; the major cleavage site was Leu^{697}–Val^{698} and the minor cleavage site, Phe^{684}–Tyr^{685}. In addition, the trichloroacetic acid-soluble supernatant of α_2-M after treatment with pepsin at pH 5.5 was found to contain a peptide, Arg–Val–Gly–Phe, in a small amount, corresponding to residues 681–684 of α_2-M, indicating the cleavage of Leu^{680}–Arg^{681}. Since the C-terminal polypeptide starting with Arg^{681} was not found, this tetrapeptide is thought to have been produced by secondary cleavage of the band I peptide.

The N-terminal amino acid sequences of the band I and II peptides produced by gastricsin at pH 5.5 and/or 6.5 were determined as follows.

Band I :

Ser–Val–Ser–Gly–Lys–Pro–Gln–Tyr––––

Band II Major :

Ala–Arg–Leu–Val–His–Val–Glu–Glu––––

Band II Minor :

Tyr–Glu–Ser–Asp–Val–Met–Gly–Arg––––

The band I peptide had the N-terminal sequence identical with that of native α_2-M[15] and the band II peptide was a mixture of one major and one minor peptide (molar ratio, 2.2:1). The results indicate that the major cleavage by gastricsin occurred at the His^{694}–Ala^{695} bond and the minor cleavage at the Phe^{684}–Tyr^{685} bond in the bait region[3] of α_2-M. The same sequences were obtained with the bands

obtained at pH 6.5 except that the band II peptide contained only the major peptide. In addition, the peptide Arg–Val–Gly–Phe was also found in a small amount in the trichloroacetic acid-soluble supernatant of α_2-M after treatment with gastricsin at pH 5.5.

Conformational Changes of α_2-M by Treatment with the Proteinases

Figure 3c shows native PAGE patterns of native α_2-M and α_2-M samples treated with pepsin and gastricsin at pH 5.5. Pepsin- and gastricin-treated α_2-M moved faster than native α_2-M. Similar results were obtained when α_2-M was treated by each enzyme at higher pH (data not shown).

The electron microscopic images of negatively stained α_2-M samples pretreated with pepsin and gastricsin (molar ratio, enzyme: α_2-M = 2:1) at pH 5.5 are shown in Fig. 4. The shape of intact α_2-M resembled a doughnut with a prominent central cavity. On the other hand, the shapes of proteinase-treated α_2-M were different from that of the intact α_2-M, and most of the α_2-M molecules looked like the letter H ($\sim 200 \times 150$ A) with a short vertical bar in the middle, as observed for other proteinases.[5,6,16–18] The shape of the α_2-M–gastricsin complex varied depending on the pH of the reaction mixture (data not shown); homogeneous H-shaped complex molecules were observed at pH 6.5 and slightly distorted H-shaped complex molecules at other pH values.

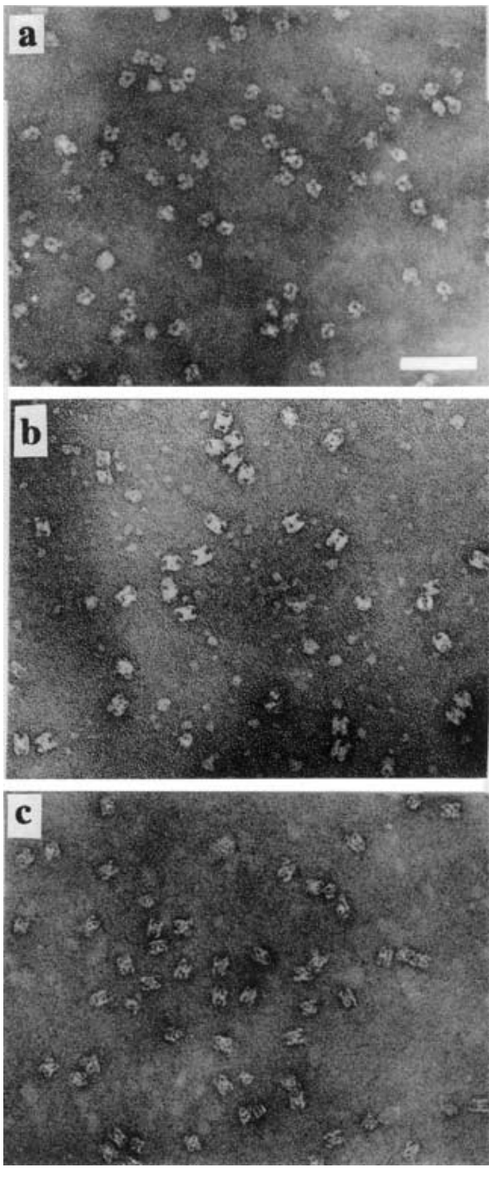

FIGURE 4 Electron micrographs of intact and proteinase-treated α_2-M. (a) α_2-M, (b) α_2-M treated with pepsin at pH 5.5. (c) α_2-M treated with gastricsin at pH 5.5. The scale bar (shown in Figure. 4a) represents 1,000 Å.

DISCUSSION

The proteolytic activities of pepsin and gastricsin toward RCm-ribonuclease A were inhibited significantly by α_2-M; gastricsin was strongly inhibited, although not completely, whereas, pepsin was partially inhibited. Therefore, α_2-M-entrapped pepsin appears to retain more activity toward RCm-ribonuclease A than α_2-M-entrapped gastricsin. On the other hand, the activities of these enzymes towards the peptide substrate oxidized insulin B-chain were scarcely inhibited. These results indicated that the active sites of these enzymes are fairly accessible to the peptide substrate, but not so much accessible to the protein substrate, suggesting the entrapment by α_2-M. The proteolytic activity toward RCm-ribonuclease A of cathepsin E ($M_r \sim 84$ K) was completely inhibited by α_2-M,[5] whereas, that of HIV protease ($M_r \sim 22$ K) was scarcely inhibited (Athauda and Takahashi, unpublished results), although its activity toward the Malony murine sarcoma virus-derived protein ($M_r \sim 65$ K) was completely inhibited by α_2-M.[6] In the present study, the proteolytic activities towards RCm-ribonuclease A of pepsin and gastricsin, which have an intermediate size ($M_r \sim 37$ K), were shown to be inhibited less strongly than that of cathepsin E under similar conditions. These results indicate that the inhibitory effect of α_2-M on the activity toward a protein substrate depends not only on the size of the protein substrate, but also on the molecular size of the proteinase trapped by α_2-M. The molecular size is thought to be correlated with the mobility of the proteinase molecule entrapped in α_2-M, thus affecting the accessibility of the enzyme active site to the scissile bonds of the protein substrate.

The major cleavage sites by pepsin (Leu^{697}–Val^{698}) and gastricsin (His^{694}–Ala^{695}) are different from each

other and also different from the cleavage site (Phe^{684}–Tyr^{685}) by other aspartic proteinases.[5,6,19] The cleavage at His^{694}–Ala^{695} in the bait region has never, to our knowledge, been reported for any other proteinases. The cleavage site specificities of pepsin and gastricsin at pH 5.5 toward α_2-M are partially consistent with those toward oxidized insulin B-chain.[7]

Upon native PAGE α_2-M treated with pepsin and with gastricsin migrated somewhat faster than native α_2-M. Similar changes in mobility on SDS-PAGE were reported for α_2-M treated with trypsin and with chymotrypsin.[4] These results suggest that α_2-M treated with proteinases is more compact than native α_2-M, thus becoming more mobile on native PAGE by a molecular sieving effect. Indeed, it was reported from a small angle X-ray diffraction study that the gyration radii of native- and proteinase-treated α_2-M samples were approximately 74 and 66 Å, respectively.[19] In the cases of pepsin and gastricsin which are acidic proteins, the isoelectric point of the α_2-M–proteinase complex should be lower than that of native α_2-M, additionally contributing to increase the anodal electrophoretic mobility.

The α_2-M is mainly present in serum, but is also found in certain tissues such as gastric mucosa.[10] On the other hand, pepsinogen and progastricsin are present in gastric mucosa, but are also known to be present in serum, seminal fluid, lung and prostate[8] and in serum and urine,[9] respectively. It is inconceivable that pepsinogen and progastrisin can be trapped by α_2-M without activation to pepsin and gastricsin, respectively, since these zymogens have practically no proteolytic activity. They are normally activated at around pH 2.0 in the gastric juice, but may be activatable at higher pH. Especially, human gastricsin is slowly activated even at pH 7.0 (Athauda and Takahashi, unpublished data). Human pepsin and gastricsin are stable below pH 6.0 and up to pH 7.0, respectively, and show some activity toward casein up to pH 6.0 and 6.5, respectively (Athauda and Takahashi, unpublished data). Therefore, if these zymogens are activated by any chance near or below pH 6.0 (pepsinogen) or pH 6.5 (progastricsin), they might be at least partly trapped by α_2-M and their proteolytic activities inhibited significantly. In order to assess the physiological significance of the inhibition, however, it seems to be also important to clarify if pepsinogen can be activated sufficiently near pH 5.5 and above and if an excess of co-existing proteins, such as the circulating protein serum albumin, affects the stability and/or the trapping by α_2-M of the enzymes. These points are open for further experimentation.

Acknowledgement

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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