IMPROVED PURIFICATION OF SLOW MOVING PROTEASE FROM HUMAN GASTRIC MUCOSA AND ITS ACTION ON THE B CHAIN OF OXIDIZED BOVINE INSULIN

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ABSTRACT

Slow moving protease (SMP), a non-pepsinogen type acid protease present in human gastric mucosa, was purified to apparent homogeneity by an improved purification procedure including chromatography on DEAE-cellulose, concanavalin A-Sepharose and Sephadex G-150. Upon DEAE-cellulose chromatography, SMP was separated into three fractions, designated SMP-I, SMP-II, and SMP-III. SMP-I was the major component and the other two appeared to be autodigestion products of SMP-I. Investigation on the specificity of action of SMP on the B chain of oxidized insulin revealed that SMP resembles cathepsin E and pepsin A in substrate specificity rather than cathepsin D and pepsin C.

Human gastric mucosa contains a non-pepsinogen type acid protease in addition to pepsinogens A and C (7, 14), and this protease was designated slow moving protease (SMP) based on its slow electrophoretic mobility towards the anode in agar gel electrophoresis (14). To elucidate the physiological role of this protease in stomach, it is first of all important to isolate and characterize it. In 1980, we purified this protease for the first time to apparent homogeneity and investigated sev-

eral properties (5). The enzyme was shown to have a molecular weight of about 85,000 and composed of two subunits of an identical size. The enzyme was shown to be a glycoprotein, containing approximately 4 glucosamine and 16 mannose residues per molecule (M_r 85,000). It had an amino acid composition generally resembling those of cathepsin D except for the lower content of basic amino acid residues, especially lysine. It also showed some similarity to pepsinogens. It had an optimal pH at 2.0-3.5 with hemoglobin as a substrate, and was strongly inhibited by aspartic protease-specific inhibitors. Unlike pepsin, the protease was stable in alkaline solution up to pH 9.0, and was rather stable to sequential acidification and neutralization. The acidification resulted in an increase in electrophoretic mobility towards the anode. Based on these results, we tentatively designated this enzyme a cathepsin D-like acid proteinase (5). In 1987, Samloff *et al.* (15) also reported on isolation and characterization of SMP from human

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Abbreviations: SMP, slow moving protease; PG, pepsinogen; Con A, concanavalin A; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; APDT, *N*-acetyl-L-phenylalanyl-3,5-di-iodo-L-tyrosine.

gastric mucosa. SMP was reported to be different from human spleen cathepsin D in various properties including immunochemical reactivity, kinetic property, sensitivity toward the protein inhibitor from *Ascaris lumbricoides*, molecular weight and cellular origin. Based on these results, they concluded that SMP is not cathepsin D, but may be cathepsin E.

In order to clarify the properties of this enzyme further, we have purified it using an improved method of purification and investigated its specificity of action on the B chain of oxidized bovine insulin together with those of rat spleen cathepsins D and E and human pepsins A and C. The results suggest that SMP is more similar in specificity to cathepsin E and pepsin A than to cathepsin D and pepsin C. Further, we have isolated two additional minor SMP components and their relationship with the major SMP has also been investigated.

MATERIALS AND METHODS

Materials

Specimens of human stomachs were generously supplied by Dr M. Ukai. They were obtained at surgery from gastric ulcer or gastric cancer patients. The resected stomachs were freed from the invaded material and stored at -80°C until use. Bovine hemoglobin was purchased from Worthington, oxidized bovine insulin B chain and porcine pepsinogen from Sigma Chemical, and *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine (APDT) from Nakarai Chemicals. DEAE-cellulose (DE-52) was obtained from Whatman Chemical Separation, Con A-Sepharose and Sephadex G-150 from Pharmacia Fine Chemicals, and Vectastain ABC Kit from Vector Laboratories. A rabbit antiserum against SMP-I was kindly prepared by Dr M. Ichinose. Rat spleen cathepsins D and E were prepared as described by Yamamoto *et al.* (20, 21), and human pepsinogens A and C as described by Kageyama and Takahashi (6).

Assay for Proteolytic Activity

The activity toward hemoglobin was assayed essentially according to Anson (2) as described previously (5) except that the assay was performed at pH 3.4. The activity toward

APDT was determined essentially according to Tang (17). The enzyme and APDT were incubated at pH 3.5 and 37°C for 2 h and the digest was analyzed with ninhydrin according to Rosen (13).

Protein Determination

The protein concentration of the enzyme solution was determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.* (8) with porcine pepsinogen as a standard.

Purification of SMP

SMP was purified by a modification of the method used previously (5). All procedures were performed at 4°C .

Step 1 Preparation of crude mucosal extract

The mucosa from 1,840 g of resected stomachs (458 g) was homogenized in 430 ml of 0.01 M sodium phosphate buffer, pH 7.4 (buffer A) in a Waring blender. The homogenate was frozen and thawed twice, and centrifuged at 15,000 g for 60 min. The precipitate was homogenized in 400 ml of buffer A in a glass-teflon homogenizer, and centrifuged at 15,000 g for 60 min. Both supernatants were combined and dialyzed against buffer A.

Step 2 First DEAE-cellulose chromatography

The dialyzed solution of the crude extract was mixed with DE-52 cellulose (about 2 l as wet volume) equilibrated with buffer A, and stirred for 1 h. The mixture was centrifuged at 10,000 g for 20 min, and the precipitate was washed three times with buffer A by centrifugation at 10,000 g for 20 min. The precipitate was packed into a glass column (9×30 cm) and the column was washed with 5 vol of buffer A and then eluted with buffer A containing 0.5 M NaCl.

Step 3 Second DEAE-cellulose chromatography

The enzyme fraction obtained in step 2 was dialyzed against buffer A, and applied to a column (3.7×84 cm) of DE-52 cellulose equilibrated with the same buffer. The column was eluted with a gradient of NaCl (0 to 0.5 M) in a total volume of 5 l. In this step, SMP was separated from pepsinogens.

Step 4 Con A-Sepharose chromatography

The SMP fraction was adjusted to

0.5 M NaCl and applied to a column (3×5 cm) of Con A-Sepharose 4 B equilibrated with buffer A containing 0.5 M NaCl. The enzyme was eluted with buffer A containing 0.4 M α -D-methylglucoside and 0.5 M NaCl. The part of the enzyme eluted unadsorbed was again treated in the same way.

Step 5 Sephadex G-150 chromatography The enzyme fraction obtained in step 4 was dialyzed against buffer A, and applied to a column (1.2×5 cm) of DE-52 cellulose equilibrated with the same buffer. The enzyme was eluted with buffer A containing 0.5 M NaCl. The concentrated sample (about 30 ml) was applied to a column (3×150 cm) of Sephadex G-150 equilibrated with buffer A, and eluted with the same buffer at a flow rate of 13 ml/h.

Step 6 Third DEAE-cellulose chromatography The enzyme fraction obtained in step 5 was applied to a column (2×52 cm) of DE-52 cellulose equilibrated with buffer A, and eluted with an NaCl gradient (0 to 0.4 M) in a total volume of 2 l.

Molecular Weight Determination

Molecular weight was determined by SDS-PAGE (19) and Sephadex G-150 gel filtration (1).

Digestion of the B Chain of Oxidized Insulin and Analysis of the Digest

The oxidized B chain of insulin (60 nmol) was digested with an about 1/100 by weight of SMP-I or SMP-II or other enzymes at pH 3.0 and 37°C for 3 h in 200 μ l of 0.5 M sodium formate buffer. In the experiments with pepsins, pepsinogens A and C were used and allowed to be activated to pepsins A and C, respectively, in the reaction mixture. About one-third of the digest was submitted to high performance liquid chromatography (HPLC) using a Toyo Soda SP-8700 or a Hitachi 655A-11 system on a column (0.4×25 cm) of TSK-Gel ODS-120T. The column was eluted with a gradient of acetonitrile (0 to 60% in 60 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The effluent was monitored by measuring absorbance at 215 nm and the peptide peak fractions were pooled separately. An aliquot from each pooled fraction was submitted to amino acid analysis.

Fig. 1 Chromatographic purification of SMP. The fractions under the bar were pooled. (a): The second DEAE-cellulose chromatography. The sample from the first step was applied to a column (3.7×84 cm) of DE-52 cellulose. Fraction size, 16 ml. (b): Sephadex G-150 chromatography. The sample from the step of Con A-Sepharose chromatography was concentrated through a DE-52 column and applied to a column (3×150 cm) of Sephadex G-150. Fraction size, 6.3 ml. (c): The third DEAE-cellulose chromatography. The sample from the step of Sephadex G-150 gel filtration was loaded on a column (2×52 cm) of DE-52 cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.4, and eluted with a gradient of NaCl (0 to 0.4 M) in a total volume of 2 l of the same buffer. Fraction size, 6.3 ml

Amino Acid Analysis

Samples for analysis were hydrolyzed with 0.3 ml of 6 N HCl containing 0.05% phenol at 110°C for 24 h in evacuated sealed tubes. The amino acids were determined with a Hitachi 835 amino acid analyzer according to Spack-

Table 1 *Purification of SMP*

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude homogenate supernatant	840	15,015	32,480	2.16	100
First DE-52 PG ^a + SMP	695	6,933	28,520	4.11	87.8
Second DE-52 SMP	745	1,676	1,584	0.94	4.88
PG	793	3,053	25,310	8.29	77.9
Con A-Sepharose SMP	775	181	1,384	7.65	4.26
Sephadex G-150 SMP	108	42	841	20.0	2.59
Third DE-52 SMP-I	195	18.9	477	25.2	1.47
SMP-II	130	7.12	183	25.7	0.56
SMP-III	100	1.29	30	23.3	0.092

^aPG, pepsinogen.man *et al.* (16).*Immuno-double Diffusion Analysis*

This was performed according to Ouchterlony *et al.* (11) on a 1% (w/v) agarose gel plate, using 0.3 to 1 μ g of each antigen. The plate was kept at 4°C for 1 to 2 days.

Enzyme-linked Immunosorbent Assay (ELISA)

The avidin-biotin-peroxidase complex technique was used with Vectastain ABC Kit according to Hsu *et al.* (3), using an antiserum against SMP-I. Each antigen was dissolved in 0.01 M sodium phosphate buffer, pH 7.4, to a concentration of 50 or 100 ng/ml. The color developed by peroxidase reaction was measured at 415 nm.

RESULTS*Purification and Properties of SMP*

SMP was purified by successive column chromatography on DEAE-cellulose, Con A-Sepharose, and Sephadex G-150 as shown in Fig. 1 and the results of a typical purification are summarized in Table 1. At the step of the third DEAE-cellulose chromatography, SMP

Fig. 2 SDS-PAGE of the purified SMP components. Lane 1, molecular weight markers: phosphorylase (M_r 97 K), bovine serum albumin (66 K), ovalbumin (45 K), and carbonic anhydrase (29 K); lanes 2-4, SMP-I, SMP-II, and SMP-III, respectively, under reducing conditions; lanes 5-7, SMP-I, SMP-II, and SMP-III, respectively, under non-reducing conditions

was fractionated into three fractions, and they were designated SMP-I, SMP-II, and SMP-III, in the order of elution from the DE-52 column. SMP-I was the major component. The specific activity toward hemoglobin was almost equal among these components (Table 1). They gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Fig. 3 Determination of the molecular weight of SMP by Sephadex G-150 gel filtration (a) and SDS-PAGE (b). Vertical arrows indicate the position of SMP. (1) in the absence of 2-mercaptoethanol; (2) in the presence of 2-mercaptoethanol. γ G, γ -globulin; PHO, phosphorylase; BSA, bovine serum albumin; OVA, ovalbumin; CA, carbonic anhydrase; STI, soybean trypsin inhibitor; MG, myoglobin; LYS, egg white lysozyme

(SDS-PAGE) under both non-reducing and reducing conditions (Fig. 2). They were hardly distinguishable in molecular weight. The molecular weight of the native protein was estimated to be about 77 K by Sephadex G-150 gel filtration (Fig. 3a). Upon SDS-PAGE in the absence of 2-mercaptoethanol, they gave a molecular weight of about 74 K whereas, in the presence of 2-mercaptoethanol, they gave a molecular weight of about 45 K (Fig. 3b).

When SMP-III was incubated for a few minutes in an acidic medium and the solution was neutralized, no loss of activity was observed unlike pepsin, as observed pre-

viously (5). On the other hand, upon incubation of SMP-I at pH 5.2 or 7.4 and 4°C for 48 h to 72 h, followed by analysis using DE-52 chromatography, small but significant amounts of SMP-II and SMP-III were produced. For instance, when a mixture of SMP-I (73%) and SMP-II (27%) was incubated at pH 5.2 and 4°C for 72 h, and then examined by DE-52 chromatography, three peaks were obtained, corresponding to SMP-I (56%), SMP-II (33%), and SMP-III (11%). Upon incubation of SMP-I at pH 3.0 and 24°C for 80 min, a more extensive conversion took place as examined by chromatography on a mono Q column (data not shown).

The antiserum against SMP-I showed clear cross-reactivity with SMP-I and SMP-II (SMP-III was not examined because of the paucity of the sample) (Fig. 4a). On the other hand, no cross-reactivity was observed with human pepsinogens A and C and rat cathepsins D and E. It was also shown that SMP-I and SMP-II as well as the other proteases examined did not cross-react with a pepsinogen C antiserum except for pepsinogen C (Fig. 4b). The above results were further confirmed by ELISA of SMP-I and other acid proteases, which showed that only SMP-I reacted with the antiserum against SMP-I (data not shown).

Substrate Specificity of SMP toward the B Chain of Oxidized Insulin

The peptides produced from the oxidized insulin B chain by the action of SMP-I, SMP-II and other related proteases could be well separated by HPLC and identified by amino acid analysis. A typical HPLC pattern obtained with SMP-I is shown in Fig. 5. The results of analysis of peptides are shown in Fig. 6 (the data for SMP-I are not shown since they were essentially the same with those for SMP-I). These results showed that the mode of action of SMP-I is significantly different from those of other acid proteases. SMP-I cleaved the peptide bonds, Leu¹¹-Val¹², Leu¹⁵-Tyr¹⁶, and Phe²⁵-Tyr²⁶ most rapidly with somewhat slower cleavages at the Glu¹³-Ala¹⁴, Tyr¹⁶-Leu¹⁷, and Phe²⁴-Phe²⁵ bonds. The cleavage pattern of SMP-I was roughly similar to those of rat cathepsin E and human pepsin A. However, rat cathepsin E cleaved the Leu¹¹-Val¹² bond more slowly and the Phe²⁴-Phe²⁵ bond more

Fig. 4 Immuno-diffusion analyses of SMP-I and SMP-II. Center well: (a) SMP-I antiserum and (b) human pepsinogen C antiserum. Satellite cells: 1, SMP-I; 2, SMP-II; 3, human pepsinogen A; 4, human pepsinogen C; 5, rat spleen cathepsin D; and 6, rat spleen cathepsin E

Fig. 5 HPLC of a 3 h-digest of oxidized insulin B chain by SMP-I. The conditions of HPLC are given in the text.

Fig. 6 Yields of peptides produced from the B chain of oxidized insulin by SMP and other acid proteases and the sites of cleavage. Bars indicate peptide fragments produced and the number in each bar shows the percent yield of the peptide. Arrowheads indicate the cleavage sites and the number under each arrowhead shows the estimated extent of cleavage in percent of the peptide bond. Large, medium and small arrowheads indicate major, secondary and minor cleavage sites, respectively.

rapidly, and additionally cleaved the Ala¹⁴-Leu¹⁵ and Leu¹⁷-Val¹⁸ bonds to some extent. On the other hand, human pepsin A showed somewhat more similar cleavage pattern to that of SMP-I except that the Gln⁴-His⁵ bond was cleaved to some extent. Rat cathepsin D and human pepsin C showed rather different peptide bond specificity from that of SMP-I. Rat cathepsin D cleaved preferentially the Leu¹⁵-Tyr¹⁶ bond followed by the Phe²⁵-Tyr²⁶ and Phe²⁴-Phe²⁵ bonds with no cleavage at the Leu¹¹-Val¹² bond. Additional minor cleavages were also observed at a few other peptide bonds. Human pepsin C cleaved preferentially the Leu¹⁵-Tyr¹⁶ and ²⁴Phe-Phe²⁵ bonds, followed by the Glu¹³-Ala¹⁴ bond and Tyr¹⁶-Leu¹⁷ bond, and did the Leu¹¹-Val¹² and ²⁵Phe-Tyr²⁶ bonds rather slowly. The specific activity of SMP-I toward *N*-acetyl-L-phenylalanyl-L-di-

dotyrosine was also different from those of other acid proteases. The relative specific activity of human pepsin A, rat cathepsin E, SMP-I, and human pepsin C was 100, 21.6, 19.2, and 12.6, respectively.

DISCUSSION

In the present study, we have purified SMP from human gastric mucosa by a modification of the previous method (5) with a nearly two-fold improvement in both overall yield and specific activity and isolated two minor components in addition to the major component as apparently homogeneous forms as examined by SDS-PAGE. The method was improved mainly by the introduction of Con A-Sepharose chromatography based on the finding of mannose and glucosamine in SMP (5).

Recently, Con A-Sepharose has also been used independently by Samloff *et al.* (15) for the same purpose although the yield and the specific activity of the purified enzyme have not been reported. The results of molecular weight determination and SDS-PAGE are essentially consistent with the previous results (5), indicating that SMP is composed of two apparently identical subunits cross-linked by a disulfide bond(s). The three components were designated SMP-I, SMP-II, and SMP-III in the order of elution in DEAE-cellulose chromatography. The SMP obtained previously (5) is thought to correspond to SMP-I. The separation of the three components on DEAE-cellulose indicates that the acidity of protein increases in the order of SMP-I, SMP-II, and SMP-III. They could not be distinguished from one another in properties such as specific activity toward hemoglobin and behavior in SDS-PAGE. However, in preliminary experiments, they gave amino acid compositions and peptide maps (prepared by digestion with *Staphylococcus aureus* V8 protease) slightly different from one another and appeared to have slightly different affinity to pepstatin (data not shown). In the amino acid compositions, especially the lysine and arginine contents appeared to decrease significantly in the order of SMP-I, SMP-II, and SMP-III and the conversion seemed to occur at a faster rate at acidic pH. This is consistent with the difference in acidity of these components. These results indicate that the three components are very similar but slightly different proteins. Upon incubation of SMP-I, it appeared to be partly converted to SMP-II and SMP-III. This is consistent with the previous results (5) that the electrophoretic mobility towards the anode increased after acid treatment possibly due to autodigestion. A similar phenomenon suggesting a molecular transition during storage has been reported recently for rat gastric mucosa SMP (9). Taken together, it appears that SMP-II and SMP-III may be further autodigestion products of SMP-I. Since the three SMP fractions gave a band of the same mobility on SDS-PAGE under reducing conditions, autodigestion, if any, is thought to occur at the peripheral part of the SMP molecule, most likely in the NH₂-terminal region. The possibility cannot be excluded, however, that the heterogeneity is produced partly due to the

presence of multiple subunits and their various combinations. Indeed, Samloff *et al.* (15) have recently reported that SMP consists of two subunits with apparent molecular weights of 42,500 and 41,000, although we did not observe such difference in subunit molecular weight. Further structural studies are necessary to clarify this point.

The cleavage pattern of oxidized insulin B chain by SMP-I resembled more those of rat cathepsin E and human pepsin A than those of rat cathepsin D and human pepsin C. The sites of cleavage by SMP-I are essentially the same as those reported for rabbit bone marrow cathepsin E (12). They are also similar to those reported for rat neutrophil cathepsin E (22) except that the latter did not hydrolyze the Leu¹¹-Val¹² bond. On the other hand, in the activity toward *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine, SMP-I resembled more rat cathepsin E than human pepsin A. Therefore, SMP-I may be said to be most similar to rat cathepsin E among the enzymes compared. Indeed, Samloff *et al.* (15) has recently demonstrated that SMP is different in several respects from cathepsin D, and Muto *et al.* (10) have reported that there is immunochemical cross-reactivity between rat gastric mucosa SMP and rat spleen cathepsin E. Yonezawa *et al.* (23) have also reported that there is an immunochemical similarity between rat gastric mucosa SMP and rat neutrophil cathepsin E. Further, Tarasova *et al.* (18) reported that an aspartic proteinase from human erythrocyte, which was tentatively classified as cathepsin E, was immunochemically indistinguishable from human gastric mucosa SMP. More recently, Jupp *et al.* (4) have reported that human gastric mucosa SMP and the aspartic proteinase from human erythrocyte membranes are catalytically equivalent to cathepsin E, using synthetic chromogenic substrates and various inhibitors. In this respect, the tentative name, *i.e.* cathepsin D-like proteinase, which we gave to SMP before (5), may not be suitable. Although, rat cathepsin E showed no cross-reactivity with the SMP-I antiserum in the present study, this may be due to the species difference between human and rat. It is necessary to elucidate the primary structures of SMP and cathepsin E to reach a definite conclusion in this regard.

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