Purification of Liver Serine Protease Which Activates Microsomal Glutathione *S*-Transferase: Possible Involvement of Hepsin

Daisuke KUNII,[1)] Miyuki SHIMOJI,[2)] Shinji NAKAMA, Masashi IKEBE, Teruyuki HACHIMAN, Izumi SATO, Atsuko TAMAKI, Kazuko YAMAZAKI, and Yoko ANIYA*

Laboratory of Functional and Molecular Pharmacology, Graduate School of Medicine, University of the Ryukyus; Okinawa 903–0215, Japan.

Received September 23, 2005; accepted February 22, 2006; published online February 24, 2006

Rat liver microsomal glutathione *S*-transferase (MGST1) is known to be activated by trypsin, however, it has not been clarified whether MGST1 is activated by a protease present in liver. In the present study we purified the MGST1 activating protease from liver microsomes and finally identified that the protease is hepsin, a type II transmembrane serine protease. When the protease was incubated with the purified MGST1 or liposomal MGST1 at 4 °C, MGST1 activity was increased 3—4.5 fold after 3—6 d. In electrophoretic and immunoblot analyses after the incubation of MGST1 with the protease MGST1 dimer and its degraded fragment were detected. These results suggest that the rat liver microsomal hepsin functions as MGST1 activating/degrading enzyme.

Key words glutathione *S*-transferase; hepsin; liver microsome; serine protease; activation

Glutathione *S*-transferases (GST, EC 2.5.1.18) are phase II drug metabolizing enzymes which catalyze the glutathione conjugation of a number of xenobiotics such as carcinogens, therapeutic drugs or toxic metabolites.[3,4)] Mammalian GSTs involve cytosolic and membrane-bound forms which are encoded by different gene families.[4,5)] Rat liver microsomal GST (MGST1), distinct from cytosolic transferases, is a homotrimer which contains one SH group per subunit and is activated by covalent binding of the SH group with *N*-ethylmaleimide[6)] or reactive metabolites formed during drug metabolism *via* cytochrome P450 system.[7,8)] MGST1 is also activated by reactive oxygen species through disulfide bond-linked dimer formation.[9—13)] Furthermore, it has been evidenced that MGST1 is activated by limited proteolysis at Lys-41 with trypsin resulting in formation of the fragment of a molecular weight (M.W.) of 12 kDa.[14)] We previously reported that MGST1 activity is increased 4—7 fold by trypsin treatment and the oxidized MGST1 with a mixed disulfide bond is more activated by the trypsin treatment and MGST1 dimer is also degraded to the fragment with a M.W. of 24 kDa.[15)]

Such a marked activation of MGST1 by trypsin prompts us to examine whether MGST1 is regulated by some protease(s) in the liver. In deed it is known that the oxidatively modified proteins become more sensitive to a proteolytic attack than native proteins and the proteolysis is regarded as a mechanism of protein metabolism or modulation of the enzyme activity.[16—19)] As preliminary experiments, we observed that MGST1 is oxidized gradually and degraded when liver microsomes were stored at a cold room. It was therefore assumed that MGST1-activating/degrading protease may be involved in liver microsomes.

In the present study, we purified the protease which can activate MGST1 and identified that the protease is hepsin. Hepsin is a type II transmembrane serine protease which is found in plasma/endoplasmic membranes[20)] and may plays a role in cell growth or coagulation.[21,22)] There are also considerable data showing a link between this protease and some types of cancer.[23,24)] However the function of the microsomal hepsin has not been clarified yet. In this study we firstly demonstrated the function of hepsin as an activating/degrading enzyme of MGST1.

MATERIALS AND METHODS

Chemicals Reduced glutathione (GSH), Triton X-100, CM Sepharose CL 6B, and calpastatin were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Lubrol PX and sodium cholate were from Nacalai Tesque (Tokyo, Japan). 1-Chloro-2,4-dinitrobenzene (CDNB), diisopropylfluorophosphate (DFP), and leupeptin were obtained from Wako Pure Chemicals (Osaka, Japan). Soybean trypsin inhibitor and Hydroxyapatite were from Mils Laboratories (Stoke Poges, Slough, U.K.) and Bio-Rad Laboratories (Richmond, CA, U.S.A.), respectively. Benzamidine-Sepharose 6B and synthetic protease substrates including *t*-butyloxycarbonyl (Boc)-Gln-Arg-Arg-4-methylcoumaryl-7-amide (MCA) were from Pharmacia-LKB Biotechnology Inc. (Tokyo, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. Anti-MGST1 antibody was prepared in our laboratory as described previously.[9)] All other reagents were of analytical grade.

Preparation of Liver Microsomes and Enzyme Assay Male Sprague–Dawley rats (300—450 g) from Nihon SLC Co. (Shizuoka) were used for all experiments. Rats were sacrificed by decapitation after overnight starvation and the liver was perfused with ice-cold 1.15% potassium chloride solution *in situ*. The liver removed was homogenized with the same solution and the microsomes were prepared by the differential centrifugation method as described previously.[13)] MGST1 activity was determined by the method of Habig *et al.*[25)] using 1 mM CDNB and 5 mM GSH as substrates. Protease activity was measured by the method of Barrett[26)] modified by Tamanoue *et al.*[27)] using a synthetic substrate Boc-Gln-Arg-Arg-MCA. Protein concentrations in microsomes and in the fraction containing purified MGST1 were measured by the methods of Lowry *et al.*[28)] and of Peterson,[29)] respectively.

* To whom correspondence should be addressed. e-mail: yaniya@med.u-ryukyu.ac.jp

Purification of Microsomal Protease and Glutathione *S*-Transferase Microsomal protease was purified according to the method of Tamanoue *et al.*[27] with minor modifications: Liver microsomes prepared from 10 rats were washed twice by centrifugation at 105000 ***g*** for 60 min after resuspension in 0.15 M Tris–HCl buffer (pH 8.0) and followed by further two washing with 0.01 M potassium phosphate buffer (pH 7.4). The four washed microsomes thus obtained were suspended in 0.01 M phosphate buffer (pH 7.4) containing 0.15 M sodium chloride, solubilized by 1% sodium cholate and further centrifuged at 105000 ***g*** for 60 min. The supernatant was applied to a Hydroxyapatite column (3×35 cm) equilibrated with 0.01 M phosphate buffer (pH 7.4, containing 0.15 M sodium chloride and 1% sodium cholate) and eluted with a liner gradient from 0.01 to 0.2 M of phosphate in the buffer. The fraction with the protease activity was concentrated by ultrafiltration (molecular cut 10000, Amicon P-10) under nitrogen gas and chromatographed on Sephadex G-100 column (1.6×77 cm). The fraction with the protease activity from the Sephadex G-100 column was dialyzed against 0.02 M triethanolamine–HCl buffer (pH 8.0) containing 0.02% lubrol and then chromatographed on a Benzamidine-Sepharose 6B column (1.6×5.5 cm) equilibrated with the same buffer. The fraction with the protease activity from the Benzamidine-Sepharose 6B column was then rechromatographed on the column and the final fraction was used as a purified protease (BZ-II). The protein concentration of each fraction was measured by the method of Lowry *et al.*[28] after dialysis against water.

MGST1 was purified as an unactivated form according to the method of Morgenstern *et al.*[30] with minor modifications. Microsomes which were prepared from the liver of 10 rats after perfusion with 1.15% potassium chloride were solubilized by Triton X-100 and then purified by using Hydroxyapatite and CM Sepharose 6B columns.

Preparation of Antihepsin Antibody Hepsin antibodies prepared by immunization of two rabbits with a synthetic peptide corresponding to amino acid 397—416 (carboxy terminus) of rat hepsin[31,32] were obtained from Peptide Institute Inc. (Osaka, Japan).

Identification of Purified Protease Molecular weight of the purified protease was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under non- or reducing conditions. The purified protease (BZ-II) was applied on a 12.5% gel followed by electrophoresis by the method of Laemmli[33] and the proteins were detected by silver staining. Immunoblot analysis of the protease was performed by using the anti-hepsin antibody.

The purified protease (BZ-II) was also analyzed by the instrument of the matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS, Bluker Daltonics, Leipzig, Germany) according to the manufactural instructions. After electrophoresis of the protease (BZ-II, 10 μg) on SDS-gel on reducing conditions, the protease was stained with coomassie brilliant blue. The stained protein (molecular weight of 31 kDa) was extracted from the gel followed by digestion with the In-Gel Digestion Kit (Millipore) and then analyzed by the MALDI-TOF MS instrument. Data were calculated using Swiss Prot database.

Preparation of Liposomal MGST1 Liposome was prepared according to the text. Phosphatidylcholine (12 mg) was dissolved in chloroform–methanol (1 : 1) in the test tube and then removed the solvents by spraying nitrogen gas followed by vacuum drying for 6 h. The lipid membrane thus obtained was mixed in Triton X-100 (120 μl) and 0.01 M phosphate buffer (pH 7.4) in 3 ml and shaken for 2 h. Triton X-100 was then removed from the micelle mixture by shaking with beads (Bio Beads SM-2 Absorbent, Bio-Rad Lab.) for 2 h followed by centrifugation. Thus the lipid/phosphate buffer micelle was used as a liposome. Liposomal MGST1 was prepared as follows: the lipid membrane (liposome) was mixed in Triton X-100 (110 μl), 1.89 ml of 0.01 M phosphate buffer (pH 7.4) and 1ml of purified MGST1 and then Triton X-100 was removed by the Bio-Beads as well. The mixture thus obtained was used as liposomal MGST1.

Effect of Purified Protease on Purified MGST1 or Liposomal MGST1 The action of the purified protease (BZ-II) on the purified MGST1 was evaluated as follows: The purified protease and MGST1 were concentrated by ultrafiltration under nitrogen gas (membrane PM 10, Amicon Inc., MA, U.S.A.) and dehydration gel (Atto, Tokyo) respectively. The two concentrated enzymes were mixed in the test tube (1 : 1 in volume, protein content 1.3 μg : 5.1 μg), and then put on room temperature for 2 h followed by store at 4 °C. The aliquot was taken at indicated times for the assay of MGST1 activity and elrctrophoretic/immunoblot analysis of the MGST1 was performed using the antibody against MGST1 as reported previously.[9]

Similarly the liposomal MGST1 (60 μl, 0.6 μg) was incubated with the concentrated protease (5 μl, 10 μg) for 2 h at room temperature and put on 4 °C. The MGST1 activity and electrophoretic analysis were examined at indicated times as well.

RESULTS

Purification of Microsomal Protease Solubilized microsomes (350 ml) was applied to a Hydroxyapatite column and eluted with a linear gradient of 0.01—0.2 M potassium phosphate. The fraction (tube number 141—189) with the protease activity eluted from the Hydroxyapatite column was chromatographed on a Sephadex G-100 and the peak with the activity thus obtained was subjected to an affinity chromatography on Benzamidine-Sepharose 6B column (Figs. 1—3). The fraction from the Benzamidine-Sepharose 6B column was chromatographed again on the Benzamidine Sepharose 6B column and the fraction was used as the purified protease (BZ-II). Recovery of the protease was summarized in Table 1. Protease activity in the final fraction (BZ-II) was 30% of solubilized microsomes and the protease was purified 14880 fold from the microsomes. The protein content of BZ-II was only 0.003% solubilized microsomes.

Purified protease (BZ-II) was concentrated with ultrafiltration up to 20 fold, and then the electrophoresis on the SDS-polyacrylamide gel was carried out. As indicated in Fig. 4, BZ-II showed a major band with 55 kDa and minor bands of 42 and 41 kDa under non-reducing conditions whereas 31 kDa or two bands with 31 and 19 kDa were detected under reducing conditions. Since hepsin shows 32 and 19 kDa in reduced conditions,[34,35] it was assumed that the purified protease is hepsin.

Properties of Purified Microsomal Protease Tables 2

and 3 indicate the effect of synthetic substrates and protease inhibitors on the purified protease BZ-II activity. BZ-II degraded the synthetic substrates with the order of Gln-Arg-Arg>Gln-Gly-Arg>Glu-Ala-Arg>Gly-Lys-Arg>Val-Pro-Arg>Leu-Lys-Arg. The protease activity was inhibited markedly by leupeptin, dithiothreitol (DTT) and moderately by $CaCl_2$, $ZnSO_4$, E-64 and NaN_3.

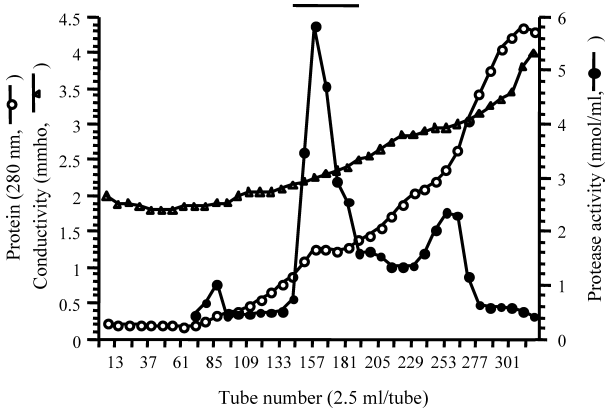

Fig. 1. Chromatography of Solubilized Microsomes on Hydroxyapatite Column

Solubilized microsomes (350 ml) were applied to a Hydroxyapatite column (3.0×35 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.5) containing 1% sodium cholate and 0.15 M NaCl (buffer A). After extensive washing with the buffer A, the sample was eluted with a linear gradient of 0.01—0.2 M potassium phosphate in the same buffer (each 500 ml) at a flow rate of 11.4 ml/h. The fractions under the bar were pooled (No. 141—189).

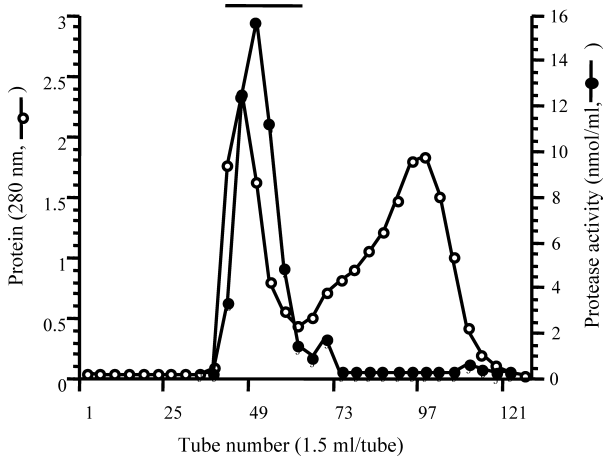

Fig. 2. Chromatography of the Hydroxyapatite Fraction on Sephadex G-100 Column

The fraction with the protease activity eluted from the Hydroxyapatite column (124 ml) was concentrated to 2.5 ml by ultrafiltration under nitrogen gas and was applied to Sephadex G-100 column (1.6×77.0 cm) equilibrated with buffer A. Gel filtration was carried with buffer A at a flow rate of 13 ml/h. The fractions under the bar were pooled (No. 41—57). The pooled sample was dialyzed against 0.02 M triethanolamine–HCl buffer (pH 8.0) containing 0.02% Lubrol PX (buffer B).

As seen in Fig. 5 of immunoblot analysis of BZ-II against the antihepsin antibody, two bands (broad band with about 48 kDa and sharp band with 31 kDa) and one band (31 kDa) were detected under non-reducing and reducing conditions, respectively. Thus the BZ-II was indicated to react with the antihepsin antibody.

The purified protease BZ-II was analyzed by MALDI-TOF MS. From analyses of protease data base (Swiss Prot), the protein score for rat hepsin was 62 and thus BZ-II was corresponded to that of hepsin with a 99% possibility. Taken all together the purified protease BZ-II was identified as hepsin.

Effects of Purified Protease on Purified MGST1 Activity Liposomal MGST1activty treated with BZ-II was increased to 238% of control (day 0) at day 2 and 291% at day 3 whereas the spontaneous increase in MGST1 activity (without BZ-II) was not observed until 3 d although the MGST1 activity was increased to 208% after 4 d and reached to almost same level as the BZ-II treated activity at day 6 (Fig. 6A). Similarly when the purified protease and MGST1 were incubated directly, as shown in Fig. 6B, MGST1 activity was increased in the presence of BZ-II to 171% at day 2, to 449% at day 6, and 399% at day 7. A spontaneous increase in the MGST1 activity (without BZ-II) was 112% at day 6 and 149% at day 7. Thus it is clear that MGST1 activity is increased by BZ-II at which the spontaneous increase in MGST1 activity has not been observed, suggesting that the purified MGST1 is activated by the purified protease BZ-II.

Electrophoretic Analysis of Purified MGST1 Treated with BZ-II Figure 7 shows the protein pattern of liposomal MGST1 treated with or without BZ-II after 8 d on SDS-gel

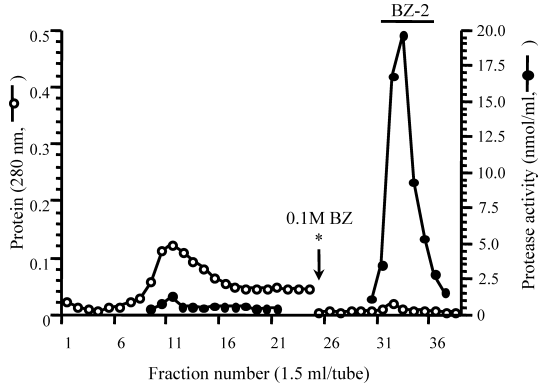

Fig. 3. Chromatography of the Sephadex G-100 Fraction on Benzamidine-Sepharose 6B Column

After dialysis the fraction eluted from the Sephadex G-100 column (38 ml) was applied to a Benzamidine-Sepharose 6B column (1.6×5.5 cm) equilibrated with buffer B. After extensive washing with buffer B followed by elution with 0.5 M NaCl the column was eluted with buffer B containing 0.1 M benzamidine–HCl (BZ) and 0.5 M NaCl at a flow rate of 10 ml/h. Each fraction was dialyzed against the buffer B to remove the benzamidine and then protease activity was determined. The fractions under the bar were pooled (No. 31—36).

Table 1. Purification of Serine Protease from Rat Liver Microsomes

Steps	Total volume (ml)	Total protein (mg)	Total activity (nmol)	Specific activity (nmol/mg)	Purification factor	Yield (%)
Cholate extract	350.0	1102.50	273.6	0.2	1	100
Hydroxyapatite	124.0	234.61	387.0	1.6	8	141
Sephadex G100	32.0	55.82	209.3	3.7	19	76
Benzamidine-Sepharose	10.5	0.73	115.6	158.3	792	42
Re Benzamidine-Sepharose	7.5	0.03	81.8	2976.0	14880	30

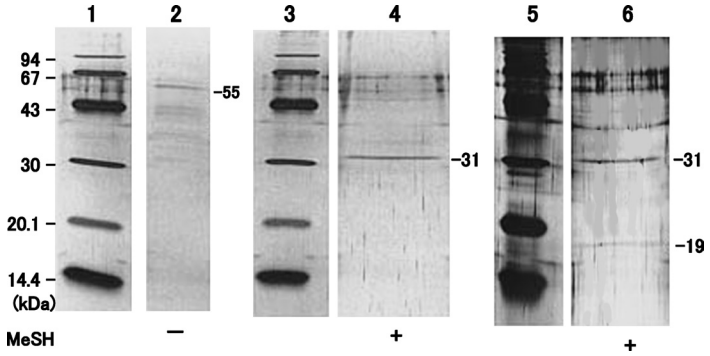

Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of Purified Protease

The purified protease (BZ-II) was subjected to SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions (involving mercaptoethanol) in a 12.5% gel followed by silver staining. Lane 1; molecular marker, lane 2; purified protease (non-reducing conditions), lane 3; molecular marker, lane 4; purified protease (Lot No. 1) (reducing conditions), lane 5; molecular marker, lane 6; purified protease (Lot No. 2) (reducing conditions), MeSH; mercaptoethanol.

Table 2. Activity of Purified Protease for Synthetic Substrates

Substrates	Activity (%)
Boc-Gln-Arg-Arg-MCA	100
Boc-Gln-Gly-Arg-MCA	58
Boc-Glu-Ala-Arg-MCA	41
Boc-Gly-Lys-Arg-MCA	25
Boc-Val-Pro-Arg-MCA	20
Boc-Leu-Lys-Arg-MCA	19
Boc-Leu-Ser-Thr-Arg-MCA	13
Boc-Ala-Gly-Pro-Arg-MCA	12
Z-Phe-Arg-MCA	9
Boc-Glu-Lys-Lys-MCA	8
Suc-Leu-Leu-Val-Tyr-MCA	2

Each substrate (final 0.1 mM) and purified protease were incubated at 37 °C for 30 min in 0.1 M Tris–HCl buffer (pH 8.0) in a total volume of 0.25 ml. Values are represented as the mean of duplicate incubations.

Table 3. Effect of Protease Inhibitors on Purified Protease Activity

Inhibitors	Concentration (mM)	Activity (%)
None		100
Leupeptin	0.1	4
TLCK	0.1	88
TPCK	0.1	87
E64	0.1	69
NEM	1	75
EDTA	1	92
DTT	10	6
NaN_3	1	66
$MgCl_2$	1	79
$CaCl_2$	1	57
$ZnSO_4$	1	41

Purified protease was preincubated in 0.1 M Tris–HCl buffer (pH 8.0) at room temperature for 30 min in the presence or absence of each inhibitor and then protease activity for Boc-Gln-Arg-Arg-MCA was measured. Values are represented as the mean of duplicate incubations. TLCK, *N*-*α*-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; MEM, *N*-ethylmaleimide.

electrophoresis. In control (without BZ-II) two proteins (18, 37 kDa) were observed (lane 3) whereas new proteins (27, 15 kDa) besides that seen in control were detected in BZ-II treated MGST1 (lane 4). The MGST1 used in Fig. 6B was also analyzed by immunoblotting. As shown in Fig. 8 only one band (17 kDa) was observed in both control at day 6 and BZ-II treated MGST1 at day 0 to day 4. However proteins with M.W. of 34 kDa and trace amount of 26 kDa were detected at day 6 in BZ-II treated MGST1, suggesting that the

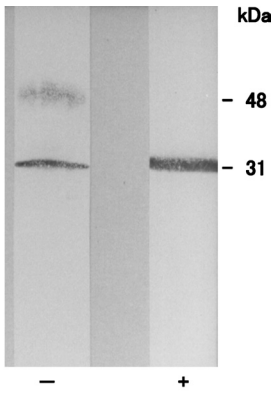

Fig. 5. Immunoblotting of Purified Protease

BZ-II fraction was concentrated by ultracentrifugation and then about 10 μg was applied to a 12.5% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to nitrocellulose membrane and detected by immunoblotting using the anti-hepsin antibody. MeSH; mercaptoethanol.

34 kDa protein is an activated form of MGST1.

DISCUSSION

Microsomal GSH *S*-transferase (MGST1) is a homotrimer (molecular weight of monomer MGST1; 17 kDa) and is activated by limited proteolysis at Lys-41 accompanying a fragment with a molecular weight of 12 kDa.[14] MGST1 monomer contains only one sufhydryl group (Cys-49) and is also activated by oxidative modification of the SH group such as disulfide bond-linked MGST1 dimer formation or a mixed disulfide bond formation.[9,10]

In the present study we purified a serine protease as MGST1 activating enzyme from rat liver microsomes. The purified protease (BZ-II fraction) showed the main band of 55 kDa on SDS-polyacrylamide gel electrophoresis in non-reducing conditions whereas both 31 and 19 kDa proteins were detected in reducing conditions. This pattern on SDS polyacrylamide gel electrophoresis is similar to that of hepsin.[31,34,35] Furthermore the BZ-II (M.W. of 31 kDa) reacted with the antihepsin antibody as seen by Western blotting and the peptide analyses of the BZ-II by MALDI-TOF MS showed a 99% similarity to that of hepsin. Thus the purified protease with 31 kDa in reducing conditions was identified as hepsin.

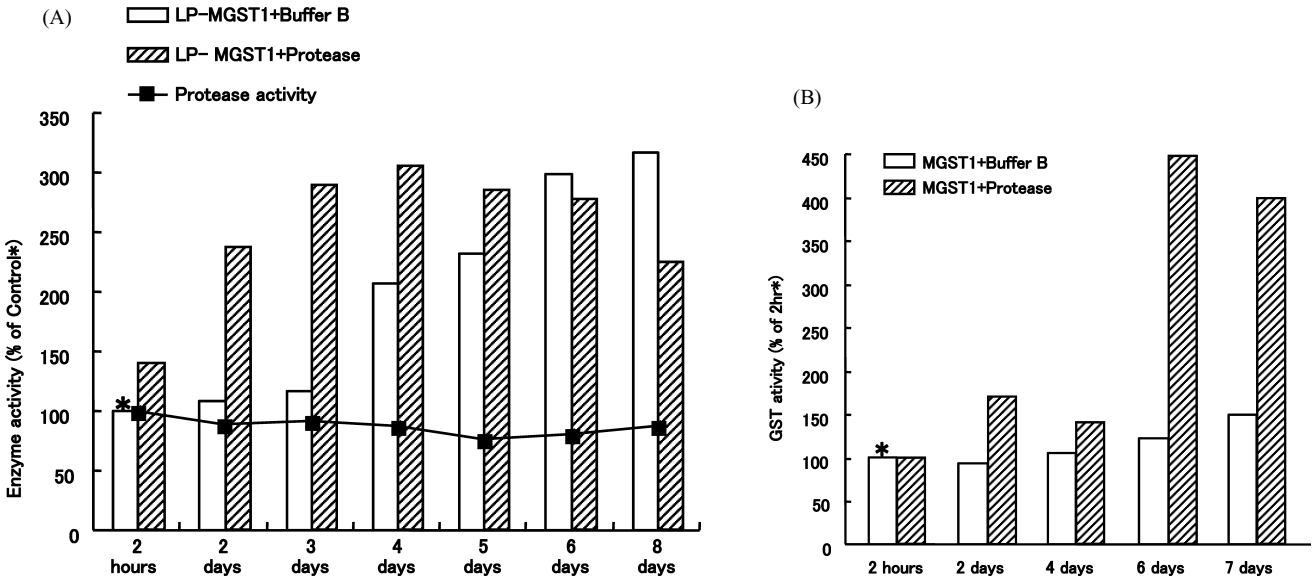

Fig. 6. The Effect of Purified Protease on MGST1 Activity

(A) Purified MGST1 was inserted in liposomal membrane (liposomal-MGST1) which was prepared under nitrogen. Purified protease (10.1 μg/5 μl) or buffer B was added to 60 μl of liposomal-MGST1 (LP-MGST1) and incubated for 2 h at room temperature followed by storage at 4 °C. Aliquot was removed at the indicated times and GST/protease activities were measured. Buffer B: 0.02 M triethanolamine–HCl containing 0.02% Lubrol PX (pH 8.0). * Control activity; GST activity in control (liposomal MGST1+buffer B) at 0 h is 9.9 μmol/mg. Protease activity of liposome+purified protease at 0 h is 1.7 μmol/mg. (B) Purified MGST1 (1.3 μg) and protease (BZ-II, 5.1 μg) concentrated by dehydration gel and ultrafiltration were incubated at room temperature for 2 h followed by store at 4 °C. Aliquot was taken at the indicated times for measuring GST activity. * Control activity; GST activity of the sample (MGST1+buffer B) after incubation for 2 h at room temperature is 5.1 μmol/mg.

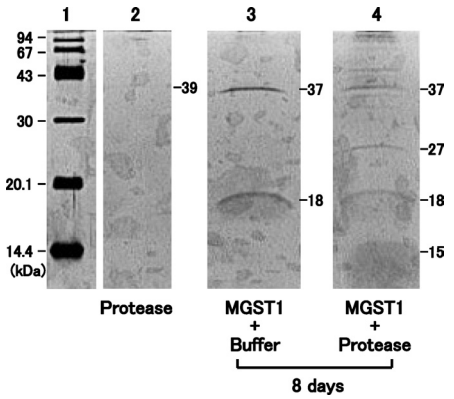

Fig. 7. Fragmentation of Liposomal-MGST1 by Purified Protease

The MGST1 samples which were used in the experiments in Fig. 6A were subjected to SDS-polyacrylamide gel electrophoresis under non-reducing conditions in a 20% gel followed by silver staining. Lane 1: molecular marker. Lane 2: liposome plus purified protease, stored at 4 °C for 8 d. Lane 3: liposomal-MGST1 plus buffer B, stored at 4 °C for 8 d. Lane 4: liposomal-MGST1 plus purified protease, stored at 4 °C for 8 d. Buffer B: 0.02 M triethanolamine–HCl containing 0.02% Lubrol PX (pH 8.0).

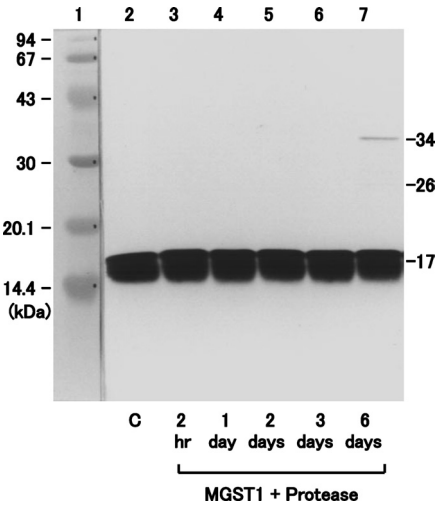

Fig. 8. Immunoblotting after Gel Electrophoresis of Purified MGST1 Incubated with Purified Protease

MGST1 samples used in Fig. 6B were subjected to SDS-polyacrylamide gel electrophoresis under non-reducing conditions in a 15% gel. Electrophoresis and immunoblotting of the MGST1 were carried out under the same conditions described in previously.[7] Lane 1; marker, lane 2(C); MGST1 plus buffer B at day 6, lane 3; MGST1 plus protease at 2 h at room temperature, lane 4—7; MGST1 plus protease at 1, 2, 3 and 6 d at 4 °C.

To clarify the function of the protease, hepsin, we examined whether the protease can activate the purified MGST1. When the liposomal MGST1 was incubated with BZ-II, the MGST1 activity was increased 2.8 fold in the presence of the BZ-II at day 3 at which the control MGST1 (without BZ-II) activity was not increased (Fig. 6A) whereas at day 6 MGST1 activity was increased 2.8 fold in both control and BZ-II treated MGST1. These data indicate that liposomal MGST1 is activated by the BZ-II until 3 d after incubation with the protease whereas after 4 d MGST1 is also activated spontaneously without the protease. Furthermore the activation of MGST1 by the protease was confirmed by direct incubation of both enzymes in which MGST1 activity was increased 4.5 fold after 6 d by the protease without spontaneous increase in control MGST1 (Fig. 6B). Since MGST1 activation by the BZ-II was observed at 2 and 3 d in liposome but at 6 d without lipid, it was suggested that MGST1 activation by the protease may be accelerated by the presence of lipid (liposome). Thus it was demonstrated that the purified protease hepsin activates MGST1 directly or in liposome.

We previously reported that when microsomes were incubated with trypsin alone, proteolytic product is a fragment with 12 kDa whereas pretreatment of microsomes with oxidants such as hydrogen peroxide or diamide followed by further treatment with trypsin causes MGST1 dimer (M.W. of 34 kDa) and its degraded product with M.W. of 24 kDa.[15]

Thus proteolytic products with 24 kDa from MGST1 dimer and 12 kDa from MGST1 monomer were designated as fragment A and B.[15)]

We tried to detect proteolytic product of MGST1 formed by BZ-II treatment. The protein with M.W. of 37 kDa was observed at day 8 in control. We often observed that MGST1 is spontaneously oxidized in microsomes to its dimer during store at 4 °C, it was therefore suggested that the 37 kDa protein is a MGST1 dimer and the MGST1 in liposome is activated by spontaneous oxidation to the dimer. On the other hand in addition to the 37 kDa protein 27 kDa and about 15 kDa proteins were also observed in the BZ-II-treated liposomal MGST1. As mentioned above, it was suggested that the 37 kDa protein is MGST1 dimer and 27 and 15 kDa proteins are products of MGST1 dimer (fragment A) and monomer (fragment B), respectively, degraded by BZ-II. Since MGST1 activity was higher in control (316%) than that of BZ-II-treated MGST1 (225%) at that time (Fig. 6A) and more MGST1 dimer was observed in control (Fig. 7), it was suggested that MGST1 is activated by forming MGST1 dimer whereas proteolysis of MGST1 dimer or monomer by BZ-II may be not or slightly contributed to the MGST1 activation. Furthermore the proteins with M.W. of 34 kDa and trace amount of 26 kDa protein were detected by immunoblotting against antiMGST1 antibody in purified MGST1 after direct incubation with BZ-II for 6 d (Fig. 8). From that the 34 kDa was not detected in control at day 6, it was confirmed that MGST1 is activated by the protease by forming MGST dimer. Taken all together these results suggest that the purified protease BZ-II stimulates MGST1 dimer formation resulting in the activation of the MGST1.

Such MGST1 dimer formation was not observed when trypsin was incubated with MGST1 (data not shown) although trypsin itself can degrade the MGST1 dimer.[15)] In addition we also found that at room temperature the hepsin makes MGST1 dimer within 30 min (unpublished data). It was therefore suggested that the hepsin preferentially makes MGST1 dimer and then degrades it. It is curious why protease hepsin activates MGST1 by its dimer formation but not by proteolysis like trypsin and how the hepsin stimulates MGST1 dimer formation.

Hepsin is a type II transmembrane serine protease found in plasma membrane or in microsmes.[20—22)] Hepsin is formed by a small N-terminal cytoplasmic region, a membrane spanning region, and an extracellular region.[20)] The extracellular portion includes a 255-residue trypsin-like serine protease domain and a 109-residue region that forms a novel, poorly conserved, scavenger receptor cysteine-rich (SRCR) domain.[36,37)] The two domains contain several intradomain disulfide bonds and are associated each other through a single disulfide bond and an extensive network of noncovalent interactions. Concerning MGST1 dimer formation by hepsin, thiol/disulfide exchange may happen between disulfide bonds in the protease and the thiol in MGST1 as seen in the treatment of MGST1 with diamide.[10)] However the mechanism of the activation of MGST1 by the protease, hepsin is remained to be clarified.

Although the biological function of hepsin is not well understood, *in vitro* and *in vivo* studies have suggested some possible roles in cell growth and blood coagulation[21,22)] and in ovarian or prostate cancer.[23,24)] Several proteases such as cytochrome P-450 degrading protease[38)] or endopeptidase[39)] have been involved in liver microsomes, however it is not clear that these proteases contribute to MGST1 activation. In consideration that minor components are still present in BZ-II fraction as seen in electrophoretic pattern (Fig. 4, lane 2), we can not exclude completely that these minor components may contribute a dimer formation or activation of MGST1. Indeed we just purified another protease which has not reacted with antihepsin antibody. It needs to confirm whether the dimer formation and activation of MGST1 is inhibited by the antihepsin antibody and to purify the protease by measuring MGST1 dimer formation. The study concerning the mechanism of MGST1 activation by microsomal protease(s) is in progress in our laboratory.

In conclusion we purified the protease which activates MGST1 from rat liver microsomes and the protease was identified as hepsin. The hepsin seems to activate MGST1 through MGST1 dimer formation although its activation mechanism is remained to be clarified.

Acknowledgements The authors wish to thank to Prof. S. Oku and Dr. H. Iwasaki of Center of Molecular Bioscience, University of the Ryukyus for allowing to use MALDI-TOF MS instruments and kind discussion. The authors also thank Ms. N. Murayama for typing the manuscript.

REFERENCES AND NOTES

1) Present address: *Planning and Evaluation Division, Science and Technology Policy Bureau, Ministry of Education, Culture, Sports, Science and Technology, Tokyo 100–8959 Japan*.
2) Present address: *Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-17177 Stockholm, Sweden*.
3) Jakoby W. B., Habig W. H., "Enzymatic Basis of Detoxication," Vol. 2, ed. by Jakoby W. B., Academic Press, New York, 1980, pp. 63—94.
4) Ketterer B., Christodoulides L. G., *Adv. Pharmacol.*, **27**, 37—69 (1994).
5) Anderson C., Mosialou E., Weinander R., Morgenstern R., *Adv. Pharmacol.*, **27**, 19—35 (1994).
6) Morgenstern R., DePierre J. W., Ernster L., *Biochem. Biophys. Res. Commun.*, **87**, 657—663 (1979).
7) Haenen G. R. M. M., Jansen F. P., Vermeulen N. P. E., Bast A., *Arch. Biochem. Biophys.*, **287**, 48—52 (1991).
8) Yonamine M., Aniya Y., Yokomakura T., Koyama T., Nagamine T., Nakanishi H., *Jpn. J. Pharmacol.*, **72**, 175—181 (1996).
9) Aniya Y., Anders M. W., *Arch. Biochem. Biophys.*, **296**, 611—616 (1992).
10) Aniya Y., Anders M. W., *Arch. Biochem. Biophys.*, **270**, 330—334 (1989).
11) Aniya Y., Naito A., *Biochem. Pharmacol.*, **45**, 37—42 (1993).
12) Aniya Y., Daido A., *Jpn. J. Pharmacol.*, **66**, 123—130 (1994).
13) Aniya Y., Anders M. W., *J. Biol. Chem.*, **264**, 1998—2002 (1989).
14) Morgenstern R., Lundquist G., Jörnvall H., DePierre J. W., *Biochem. J.*, **260**, 577—582 (1989).
15) Shimoji M., Aniya Y., Anders M. W., *Biol. Pharm. Bull.*, **19**, 209—213 (1996).
16) Davies K. J. A., *J. Biol. Chem.*, **262**, 9895—9901 (1987).
17) Saari H., Suomalainen K., Lindy O., Konttinen Y. T., Sorsa T., *Biochem. Biophys. Res. Commun.*, **171**, 979—987 (1990).
18) Pigeolet E., Remacle J., *Free Radic. Biol. Med.*, **11**, 191—195 (1991).
19) Strack P. R., Waxman L., Fagan J. M., *Biochem. Biophys. Res. Commun.*, **219**, 348—353 (1996).
20) Leytus S. P., Loeb K. R., Hagen F. S., Kurachi K., Davie E. W., *Biochemistry*, **27**, 1067—1074 (1988).
21) Torres-Rosado A., O'Shea K. S., Tsuji A., Chou S.-H., Kurachi K., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7181—7185 (1993).
22) Kazama Y., Hamamoto T., Foster D. C., Kisiel W., *J. Biol. Chem.*, **270**, 66—72 (1995).

23) Tanimoto H., Yan Y., Clarke J., Korourian S., Shigemasa K., Parmley T. H., Parham G. P., O'Brien T. J., *Cancer Res.*, **57**, 2884—2887 (1997).
24) Magee J. A., Araki T., Patil S., Ehrig T., True L., Humphrey P. A., Catalona W. J., Watson M. A., Milbrandt J., *Cancer Res.*, **61**, 5692—5696 (2001).
25) Habig W. H., Pabst M. J., Jakoby W. B., *J. Biol. Chem.*, **249**, 7130—7139 (1974).
26) Barrett A. J., *Biochem. J.*, **187**, 909—912 (1980).
27) Tamanoue Y., Takahashi T., Takahashi K., *J. Biochem.*, **113**, 229—235 (1993).
28) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
29) Peterson G. L., *Anal. Biochem.*, **83**, 346—356 (1977).
30) Morgenstern R., Guthenberg C., DePierre J. W., *Eur. J. Biochem.*, **128**, 243—248 (1982).
31) Kurachi K., Torres-Rosado A., Tsuji A., *Method Enzymol.*, **244**, 100—114 (1994).
32) Farley D., Reymond F., Nick H., *Biochem. Biophys. Acta*, **1173**, 350—352 (1993).
33) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
34) Zhukov A., Hellman U., Ingelman-Sundberg M., *Biochim. Biophys. Acta*, **1337**, 85—95 (1997).
35) Matsuzaki H., Jeohn G. H., Iwamatsu A., Tamanoue Y., Kim Y. T., Takahashi T., Takahashi K., *Protein & Peptide Lett.*, **4**, 55—62 (1997).
36) Somoza J. R., Ho J. D., Luong C., Ghate M., Sprengeler P. A., Mortara K., Shrader W. D., Sperandio D., Chan H., McGrath M. E., Katz B. A., *Structure* (Camb), **11**, 1123—1131 (2003).
37) Sarrias M. R., Gronlund J., Padilla O., Madsen J., Holmskov U., Lozano F., *Crit. Rev. Immunol.*, **24**, 1—37 (2004).
38) Zhukov A., Werinder V., Ingelman-Sundberg M., *Biochem. Biophys. Res. Commun.*, **197**, 221—228 (1993).
39) Ma Y. T., Rando R. R., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6275—6279 (1992).