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Purification and characterization of hepsin from rat liver microsomes

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Abstract

Hepsin, a putative cell-surface serine proteinase, has been isolated from the microsomal membranes of rat liver and purified to homogeneity by hydroxyapatite, DEAE-Sepharose, and benzamidine-Sepharose chromatography. The course of purification was monitored using antibodies raised against a 20-mer peptide at the C-terminus of rat hepsin, and the identity of the purified protein was confirmed by partial amino-acid sequencing. A single-chain precursor of ca. 50 kDa found in the microsomes underwent spontaneous maturation in the course of purification so that the last, affinity chromatography, step recovered only the mature form which dissociated to subunits of 31 and 19 kDa under reducing SDS-PAGE. Proteinase digestion experiments with microsomal vesicles are consistent with the luminal orientation of the precursor C-terminus, which would result in its extracellular orientation upon transportation to the cell surface. [^{3}H]diisopropylfluorophosphate covalently binds to the large subunit showing it to be the catalytic one. The N-terminal sequencing of this subunit demonstrates that the zymogen is converted to the active serine proteinase by cleavage at the Arg^{161}-Ile^{162} site. Activity measurements with short synthetic peptides show that the enzyme cleaves after basic amino-acid residues, Arg being preferable to Lys. The inhibition pattern is typical of trypsin-like serine proteinases. The pH-dependence of activity within the range pH 6–9 has no maximum, the activity increasing continuously with pH. These results are consistent with the earlier predictions based on hepsin amino-acid sequence and elucidate the specificity and other earlier unknown enzymatic and molecular properties of the enzyme.

Keywords: Hepsin; Protein degradation; Endoplasmic reticulum; Serine proteinase; (Rat liver microsome)

1. Introduction

Different intracellular compartments contain a number of membrane-incorporated proteinases whose main functions are believed to be preprotein processing [1–3] or cleavage of signal peptides [4,5]. Plasma membrane proteinases can be essential for functions such as cell proliferation [6], tumor growth [7], and cleavage of circulating proteins such as peptide hormones [8]. A number of membrane proteinases have been found, however, whose biological roles are still unclear [9–12].

Hepsin was first identified by screening a human liver cDNA library with a synthetic oligonucleotide probe corresponding to a highly conserved region found in many serine proteinases close to the active site [13]. By homology to other serine proteinases, its

Abbreviations: Boc, *t*-butyloxycarbonyl; Cbz, carbobenzoxy; iPr_2P-F, diisopropylfluorophosphate; Mec, 4-methylcoumaryl-7-amide; $PhMeSO_2F$, phenylmethanesulfonyl fluoride

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deduced 417 amino-acid sequence was suggested to represent a zymogen whose activation supposedly occurs by cleavage of the Arg^{162}-Ile^{163} bond to give an active proteinase, with its two subunits held together by a disulfide bond. A very hydrophobic domain found near the N-terminus was proposed a role of a membrane anchor, and hepsin, therefore, was suggested to be a membrane-bound enzyme. Later, it was indeed found by immunoblot analysis in the plasma membranes of human hepatoma and baby hamster kidney cell lines, with its catalytic C-terminal part exposed to the cell surface [14]. The protein, however, appeared as a 51 kDa band even under reducing conditions meaning that the detected form represented the zymogen. The mature, two-subunit, form of hepsin has never been detected and none of the forms has been isolated and purified so far.

Recently, hepsin was shown to be essential for the normal growth and morphology of several mammalian cell lines [15] as well as proposed a role in initiating blood coagulation on the cell surface [16]. Despite the accumulating evidence on its physiological importance, little is known about the enzymatic properties of hepsin. Here we report on the isolation and purification of hepsin from rat liver microsomal membranes, which has allowed a partial molecular and enzymatic characterization of the enzyme.

2. Materials and methods

2.1. Materials

Hydroxyapatite Bio-Gel HT and protein A conjugated horseradish peroxidase were purchased from Bio-Rad. DEAE-Sepharose CL6B was from Pharmacia. Freund's adjuvant was from Difco. Hybond-C nitrocellulose membranes and enhanced chemiluminescence kit were from Amersham. Lubrol 17A17 was from Serva. [1,3-^{3}H]iPr_2P-F was from NEN. Proteinase K was from Boehringer Mannheim. Cbz-Ala-Ala-Arg-NH-Mec was from Bachem. All other proteinase inhibitors, peptide substrates as well as *p*-aminobenzamidine Sepharose 6B were from Sigma. Custom peptide synthesis and conjugation to keyhole limpet hemocyanin were carried out at Neosystems Laboratoire.

2.2. Antibodies

Antibodies were raised against a synthetic peptide corresponding to amino acids 397–416 (carboxy terminus) of rat hepsin [17]. An additional tyrosine was attached to the amino terminus of the peptide, and it was conjugated to keyhole limpet hemocyanin via bis-diazobenzidine. On day 1 a rabbit was injected subcutaneously with a 1:1 mixture of the conjugate containing 1 mg peptide and complete Freund's adjuvant and on days 20 and 35 boosted with the same amount of the conjugate in incomplete Freund's adjuvant. Ten days after the second boost the rabbit was bled from the ear vein to obtain the immune serum. The serum was incubated with 2.7 mg/ml keyhole limpet hemocyanin for 1 hour at room temperature and the precipitate separated by centrifugation and discarded. The resulting serum was used in immunoblot analysis without further purification.

2.3. Hepsin immunoassay

The samples were subjected to SDS-PAGE in a Bio-Rad Mini-Protean II cell and transfered onto a nitrocellulose membrane in the Towbin buffer system [18]. After completion of transfer the membrane was dried, resoaked in 50 mM Tris, 0.2 M NaCl, 0.05% Tween 20, pH 7.4 (Tris/NaCl/Tween 20), blocked in Tris/NaCl/Tween 20 containing 5% non-fat dry milk for 1 h, incubated for 2 h with the antiserum and then for 1 h with protein A conjugated horseradish peroxidase both diluted 1:1000 in Tris/NaCl/Tween 20 containing 1% milk. The bands were visualized using enhanced chemiluminescence kit. Assignment of bands to hepsin was confirmed in competition experiments with the peptide used to raise the antibodies.

2.4. Purification of hepsin

Microsomal membranes. Microsomes were isolated from the livers of male Sprague–Dawley rats essentially as described previously [19]. The animals were denied food 24 h prior to sacrifice by decapitation. The livers were perfused in situ with cold 10 mM Tris-HCl, 1.14% KCl, 10 mM EDTA (pH 7.4) removed, minced, and homogenized by seven strokes

of a Potter–Elvehjem homogenizer in a twofold excess (v/w) of the same buffer. Homogenization and all subsequent isolation and purification procedures were performed at 4°C. The homogenate was centrifuged for 20 min at $10\,000 \times g$, the pellet discarded, and the supernatant centrifuged for 60 min at $105\,000 \times g$. To remove adsorbed protein the microsomal pellet was resuspended to the initial volume in 0.1 M potassium pyrophosphate (pH 7.4) and centrifuged again at $105\,000 \times g$ for 60 min. The luminal content was released from the microsomes by treatment with sodium deoxycholate [20]. The microsomes were resuspended in 50 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$ (pH 7.5) to a protein concentration of 4 mg/ml and 0.5% sodium cholate was added dropwise to a final concentration of 0.05%. After 30 min on a stirrer the microsomal membranes were pelleted by centrifugation at $105\,000 \times g$ for 60 min.

Extraction. Microsomal membranes were resuspended in 10 mM potassium phosphate buffer (pH 7.5) containing 0.15 M NaCl and 1% sodium cholate (buffer A) to a concentration of 3 mg/ml. The mixture was stirred for 1 h and centrifuged at $105\,000 \times g$ for 60 min to remove insoluble material.

Chromatography. In a typical purification cholate extract obtained from 20 rats and containing 1076 mg protein was applied on a 18×4.8 cm hydroxyapatite column equilibrated with buffer A. The column was washed with 700 ml of buffer A and eluted with a linear 0.01–0.3 M potassium phosphate gradient in 3 l of buffer A at a flow rate of 190 ml/h. Fractions of 16 ml were collected and their hepsin content was estimated by immunoblot analysis. Hepsin-containing fractions were pooled, supplied with 0.06% Lubrol, dialyzed against 10 mM Tris-HCl, pH 8, containing 0.06% Lubrol (buffer B), and applied onto a 10.5×4.8 cm DEAE-Sepharose column equilibrated with buffer B. The column was washed with 0.3 l of buffer B and eluted with a linear 0–0.3 M NaCl gradient in 2 l of buffer B at a flow rate of 85 ml/h. Fractions of 16 ml were collected and assayed for hepsin. Hepsin-containing fractions were pooled and applied on a 5×1.4 cm benzamidine-Sepharose column equilibrated with buffer B containing 0.1 M NaCl. The column was washed with ten bed volumes of buffer B containing 0.5 M NaCl and 10 mM CHAPS and eluted with the same buffer supplied with 0.1 M benzamidine. Fractions of 3 ml were collected, assayed for hepsin by immunoblot, and those containing hepsin were pooled and dialyzed three times against 500 volumes of buffer B to ensure complete removal of benzamidine which is inhibitory to trypsin-like proteinases. Activity of the sample was determined after each dialysis step. The third dialysis did not lead to a further increase in activity indicating that the inhibitor was efficiently removed.

Protein assay. The amount of protein was determined by the method of Lowry [21]. In the pure hepsin preparation, where protein content was too low, it was estimated by the density of the hepsin band on a silver-stained SDS-PAGE gel using bovine carbonic anhydrase as a standard.

Densitometry. Band densities on photographic films and polyacrylamide gels were measured on a Personal Densitometer (Molecular Dynamics).

2.5. Proteolytic digestion and amino-acid sequencing

Preparation of purified hepsin obtained from 20 rats was concentrated 15-fold in a Centricon 10 concentrator (Amicon). The preparation was treated with 20 mM 2-mercaptoethanol for 2 h and then with 50 mM iodoacetamide for another 2 h at room temperature and subjected to SDS-PAGE in a 12% gel. The gel was stained with Coomassie brilliant blue R-250 and the hepsin band excised. The material in the band was then subjected to 'in-gel' digestion with trypsin as described by Hellman et al. [22]. Briefly, the gel piece was washed with 0.2 M ammonium bicarbonate/50% acetonitrile, then completely dried. Upon reswelling with ammonium bicarbonate, 0.5 μg of modified trypsin (Promega, Madison, WI) was added and the mixture incubated at 30°C overnight. The generated peptides were extracted with 0.1% trifluoroacetic acid/60% acetonitrile and isolated by reverse-phase liquid chromatography on a μRPC C2/C18 SC 2.1/10 column operated in SMART system (Pharmacia, Uppsala, Sweden).

For automated sequencing, peptide fractions were loaded in an Applied Biosystems model 470A sequencer which was operated according to manufacturer's instructions. In the case of analysis of N-terminus of intact hepsin, the sequencing was performed on material blotted from SDS-PAGE gel onto a poly(vinylidene difluoride) membrane according to Matsudaira [23].

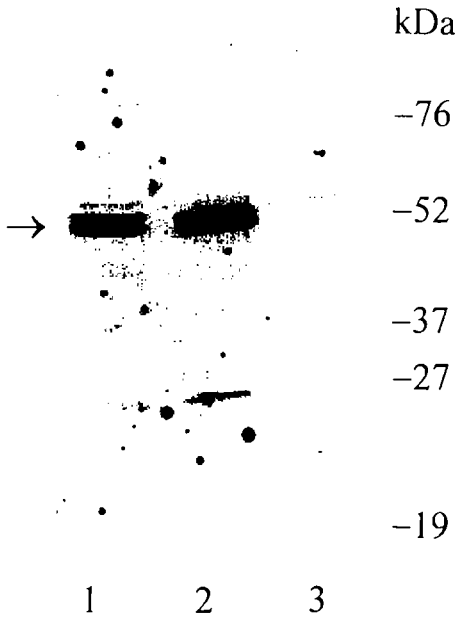

Fig. 1. Immunoblot assay of rat liver microsomes for hepsin. 20 μg of protein from microsomal fraction (1), microsomal membranes (2), and microsomal luminal content (3) was subjected to SDS-PAGE under reducing conditions in a 12% gel followed by immunoblot analysis. The arrow points to the hepsin band.

2.6. Assay of catalytic activity

Unless otherwise indicated, hepsin activity was measured in a 0.5 ml reaction mixture containing 10 mM Tris-HCl (pH 8) 0.2 mM Boc-Gln-Arg-Arg-NH-Mec and ca. 50 units of the enzyme sample or 20 μl of chromatography fractions. The incubation was started by the addition of the substrate, carried out at 37°C for 30 min, and terminated by the addition of 0.17 ml of 0.45 M $ZnSO_4$ and 0.072 ml of saturated $Ba(OH)_2$. Precipitate was separated by centrifugation and fluorescence measured in supernatant at the excitation and emission wavelengths of 380 and 460 nm, respectively. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 pmol of Mec per minute.

2.7. [^{3}H]iPr_2P-F labelling

Ca. 500 units of purified hepsin preparation was incubated with 10 μCi of [^{3}H]iPr_2P-F for 2 h at 37°C followed by SDS-PAGE under reducing conditions in a 12% gel. The gel was soaked in Amplify (Amersham), dried and subjected to autoradiography.

2.8. Proteolytic digestion of microsomes

Microsomes (3 mg/ml microsomal protein) were incubated in 50 mM Tris-HCl (pH 8), 10 mM $CaCl_2$, for 30 min at 37°C in the presence and absence of 20 μg/ml proteinase K and 1% Triton X-100. The reaction was stopped by the addition of $PhMeSO_2F$ to a final concentration of 5 mM. After 10 min on ice the samples were subjected to SDS-PAGE under reducing conditions in a 12% gel followed by immunoblot analysis.

3. Results

3.1. Purification of hepsin

Hepsin was initially shown to be present in the plasma membrane nuclei, and mitochondria and absent from cytosol [14,24]. Fig. 1 shows that hepsin is also present in the membranes, but not lumen, of the microsomal fraction as a band of about 50 kDa under

Table 1
Purification of hepsin

	Total protein (mg)	Total activity (units · 10^{-3})	Specific activity (units/mg)	Purification factor	Yield (%)
Microsomal membranes	1760	113	64	1	100
Cholate extract	1076	355	330	5.2	314
Hydroxyapatite	218	218	1000	15.6	193
DEAE-Sepharose	40	414	10350	161	366
Benzamidine-Sepharose	< 0.01	107	> 10665000	> 166000 (> 2600) [a]	95 (1.2) [a]

[a] The purification factor and yield given in parentheses were estimated by measuring hepsin immunoblot band densities in the samples of microsomal membranes and purified hepsin and relating them to protein concentrations and total protein contents in the respective fractions.

reducing conditions which corresponds, obviously, to the uncleaved hepsin precursor. This finding enabled us to use microsomal membranes as the starting material in hepsin purification. To monitor purification, antibodies were raised against the 20-mer fragment at the C-terminus or rat hepsin [17]. Antibodies against the corresponding fragment of human hepsin have earlier been shown to be highly specific for the

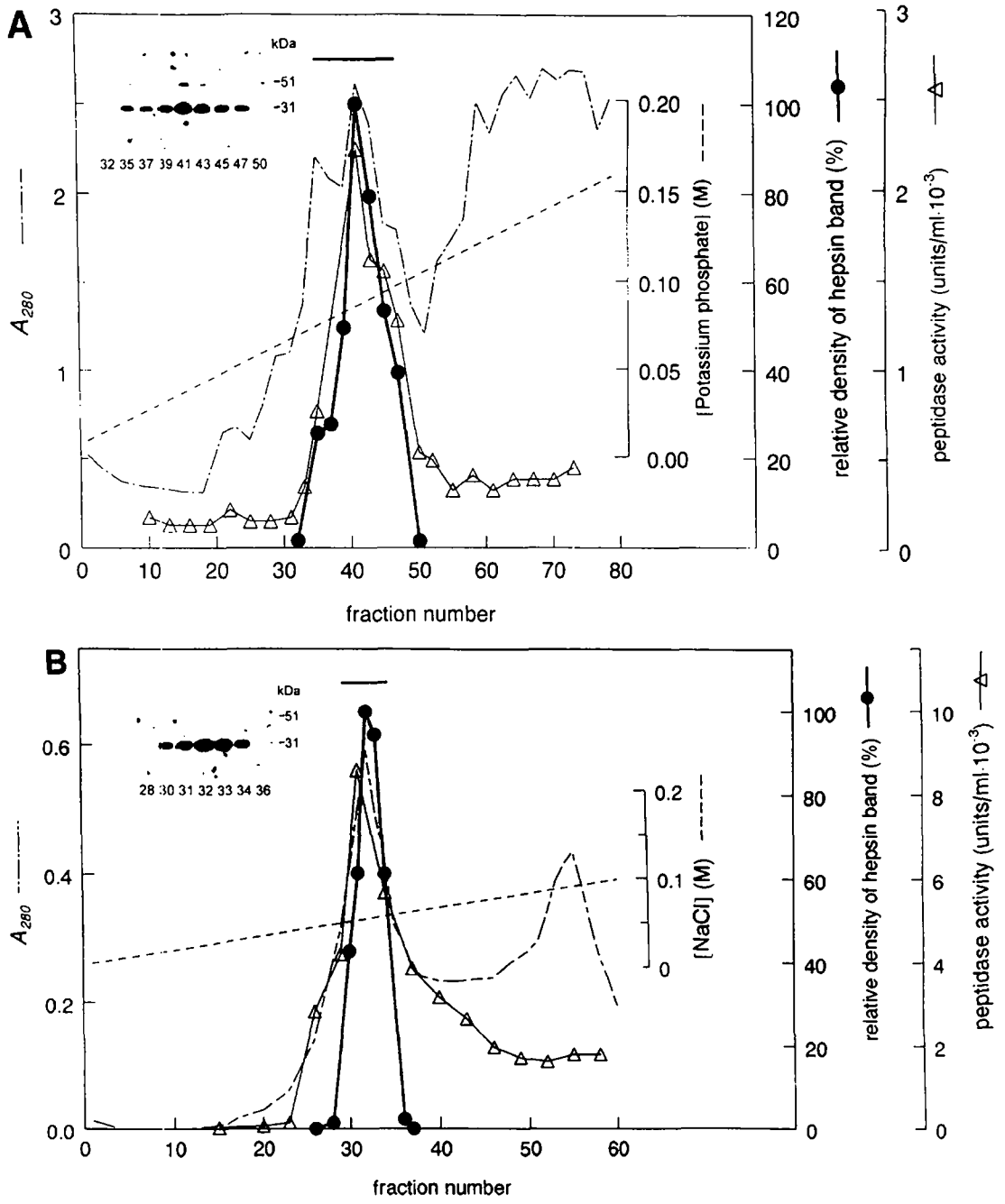

Fig. 2. Hydroxyapatite (A) and DEAE-Sepahrose (B) chromatography steps in hepsin purification. Fractions under the bars were pooled. Insets: immunoblot patterns of hepsin-containing fractions. 20 μl of each fraction was subjected to SDS-PAGE under reducing conditions in a 10% gel followed by immunoblotting. Given below are fraction numbers. No hepsin immunoreactivity was detected in fractions to the left and right of the presented region (not shown).

Table 2
Activities of purified hepsin towards synthetic substrates

Substrate	Activity (%)
Boc-Gln-Arg-Arg-NH-Mec	100
Boc-Gln-Ala-Arg-NH-Mec	30
Cbz-Ala-Arg-Arg-NH-Mec	21
Boc-Gly-Lys-Arg-NH-Mec	20
Boc-Val-Pro-Arg-NH-Mec	13
Boc-Val-Leu-Lys-NH-Mec	2.3
Boc-Glu-Lys-Lys-NH-Mec	1.7
Cbz-Arg-NH-Mec	0.5
suc-Ala-Ala-Ala-NH-Mec	0.5
suc-Ala-Ala-Phe-NH-Mec	0.3
suc-Leu-Tyr-NH-Mec	0

Activities were measured with 0.2 mM peptide substrates as described in Section 2.

proteinase [14]. The course of a typical purification is documented in Table 1 and illustrated in Fig. 2. The activity data were obtained after completion of purification, when aliquots from different purification steps were assayed for activity against Boc-Gln-Arg-Arg-NH-Mec which proved to be the best artificial substrate for pure hepsin (see Table 2 below). The peak of peptidase activity towards this substrate coincides with the peak of hepsin content (Fig. 2) indicating that assay using this peptide is quite specific for hepsin. Still, one cannot completely rule out some contribution of other microsomal proteinases to hydrolysis of Boc-Gln-Arg-Arg-NH-Mec at intermediate purification steps. That might result in slightly underestimated purification factor and yield in terms of activity. Starting with 1.8 g of microsomal membrane protein we obtained less than 10 μg of electrophoretically homogenous hepsin with an apparent purification factor of greater than $16 \cdot 10^4$ in terms of activity. This figure, however, appears to be strongly overestimated due to the activation of hepsin in the course of purification. As seen from Table 1, at least 3-fold activation occurs at the cholate extraction and further twofold at the DEAE-Sepharose chromatography steps. Activation is accompanied by changes in hepsin immunoblot pattern (Fig. 3). A strong band appears at about 31 kDa after cholate solubilization of microsomal membranes, while the 50 kDa band becomes very weak after the hydroxyapatite and disappears after DEAE-Sepharose column. This can result from proteolytic activation with the formation of a two-subunit active proteinase, with the subunits held together by a disulfide bond. To obtain more realistic estimates for the purification factor and yield the densities of the hepsin-specific bands on the immunoblots of the microsomal membranes and the final preparation were compared (not shown). This produced much more moderate figures of $2.6 \cdot 10^3$ and 1.2% for the purification factor and yield, respectively. Using these figures hepsin precursor content in the microsomal membranes can be estimated to be in the range of several picomoles per mg membrane protein.

3.2. Purity and molecular weight

The final preparation gives two protein bands, 32 and 19 kDa, on SDS-PAGE under reducing conditions (Fig. 4A). For reasons which are not clear, the sensitivity of silver staining proved to be insufficient for the analysis of the same sample under non-reducing conditions. However, we were able to circumvent this obstacle by using immunoblot in combination with enhanced chemiluminescence detection. This

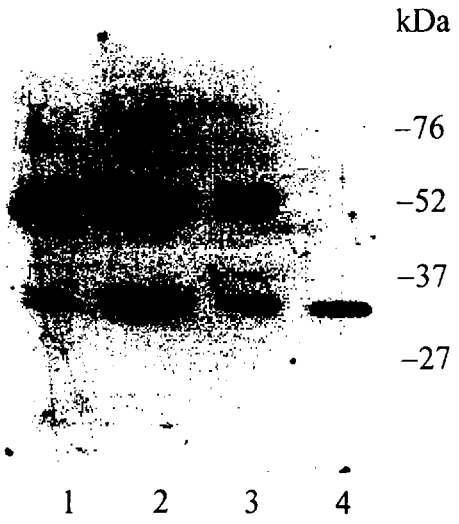

Fig. 3. Maturation of hepsin precursor in the course of purification. The samples were subjected to SDS-PAGE under reducing conditions in a 10% gel followed by immunoblot analysis. Lane 1, microsomal membranes (20 μg of protein); lane 2, cholate extract (20 μl); lane 3, pool after hydroxyapatite chromatography (20 μl); lane 4, pool after DEAE-Sepharose chromatography (20 μl).

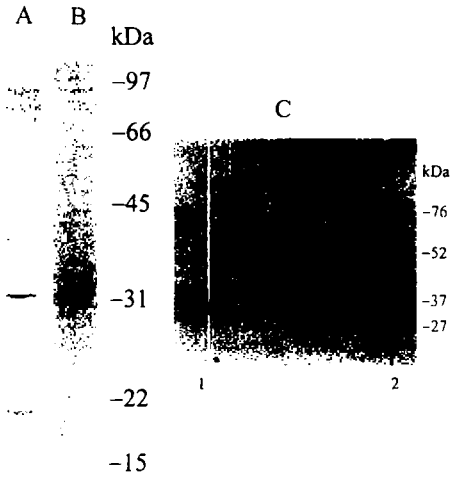

Fig. 4. Molecular properties of purified hepsin. Purified hepsin (500 units) was subjected to SDS-PAGE in a 12% (A, B) or 10% (C) gel. (A) Silver staining (reducing conditions), (B) labelling with [^{3}H] iPr_2*P*-F followed by autoradiography (reducing conditions), (C) immunoblot after SDS-PAGE under reducing (lane 1) and non-reducing (lane 2) conditions.

technique revealed a single band of 32 kDa under reducing and a single band of about 50 kDa under non-reducing conditions (Fig. 4C). Interestingly, even in this case the same sample gave a remarkably weaker signal under non-reducing conditions.

3.3. Labelling with [^{3}H]iPr_2 p-F

Incubation of purified hepsin with [^{3}H]iPr_2*P*-F followed by SDS-PAGE under reducing conditions and fluorography (Fig. 4B) revealed that the label binds to the 32 kDa subunit. This confirms that hepsin is a serine proteinase, with its large subunit being the catalytic one.

3.4. Partial sequencing and processing site

To make sure that the isolated protein is indeed hepsin, the 32 kDa subunit was subjected to partial sequencing. After reducing SDS-PAGE, the 32 kDa band was subjected to in-gel digestion followed by reverse-phase chromatography which resolved approx. 20 peptides from the digest. Two of the peptides were partially sequenced to give the sequences

VCTVTGWGNTQFYGQQ

and

VPIISNEV

which are identical to fragments 289–304 and 313–320 within the deduced amino-acid sequence of rat hepsin precursor [17]. In an additional experiment the 32 kDa band was subjected to partial N-terminal sequencing without digestion to give the sequence

IVGGQDSSLG

which is identical to fragment 162–171 within the sequence of the hepsin precursor. These data confirm that the isolated protein is hepsin and show that hepsin precursor is activated through the cleavage of the Arg^{161}-Ile^{162} bond.

3.5. Substrate specificity

To characterize the substrate specificity of hepsin it was assayed for activity towards a series of fluorogenic peptides (Table 2). In agreement with its being a member of the trypsin family, hepsin cleaves after basic amino-acid residues, with Arg strongly preferred to Lys. No cleavage after aromatic and aliphatic residues is observed. The occupancy of the S_2 site is an absolute requirement for catalysis, and another basic residue here is preferable at least to aliphatic. The nature of the residue at S_3 site also affects hydrolysis, with Gln being much more favourable than Ala.

3.6. pH Dependence of activity

The pH dependence of activity towards Boc-Gln-Arg-Arg-NH-Mec is shown in Fig. 5. No maximum

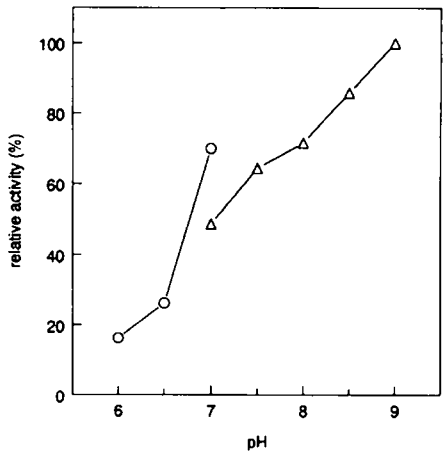

Fig. 5. pH-Dependence of hepsin activity. Activities were measured in 0.1 M Bistris (○) and 0.1 M Tris-HCl (△).

is observed within the range pH 6–9, the activity increasing continuously with pH.

3.7. Effect of inhibitors

The effect of proteinase inhibitors on hepsin activity is summarized in Table 3. Inhibition by iPr_2P-F, $PhMeSO_2F$, and 3,4-dichloroisocoumarin is consistent with the serine proteinase nature of hepsin. Of other serine proteinase inhibitors only those specific for trypsin-like enzymes (antipain, N^{α}-tosyl-L-lysine chloromethyl ketone, soybean trypsin inhibitor, leupeptin) are effective, while those active towards chymotrypsin-like (N^{α}-tosyl-L-phenylalanine chloromethyl ketone, chymostatin) or elastase-like (elastatinal) proteinases show little activity. Partial inhibition is also caused by the thiol group-directed agents dithiothreitol, E-64, and *p*-chloromercuribenzoate. At a concentration of 1 mM, calcium and magnesium exhibit a moderate and zinc a strong inhibitory effect.

Table 3
Effect of inhibitors and metal ions on activity of purified hepsin

Inhibitor	Concentration	Activity (%)
None		100
iPr_2P-F	1 mM	44
	5 mM	0
$PhMeSO_2F$	1 mM	20
3,4-Dichloroisocoumarin	0.1 mM	6
Antipain	0.1 mM	12
Leupeptin	0.1 mM	1
N^{α}-tosyl-L-lysine chloromethyl ketone	0.1 mM	54
Soybean trypsin inhibitor	0.2 mg/ml	41
N^{α}-tosyl-L-phenylalanine chloromethyl ketone	0.1 mM	81
Chymostatin	0.1 mM	85
Elastatinal	0.1 mM	91
E-64	0.1 mM	44
EDTA	1 mM	100
1,10-Phenanthroline	1 mM	83
Pepstatin A	0.1 mM	121
Dithiothreitol	1 mM	32
p-Chloromercuribenzoate	1 mM	54
$CaCl_2$	1 mM	69
$MgCl_2$	1 mM	77
$ZnSO_4$	1 mM	26

Hepsin was preincubated with the inhibitors for 30 min at room temperature, and the activities were measured as described in Section 2.

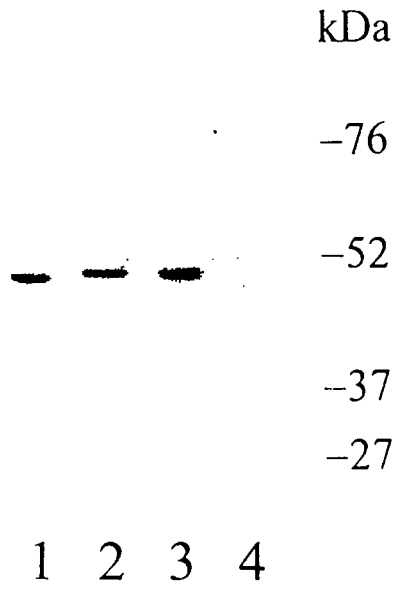

Fig. 6. Effect of proteolytic digestion on microsomal hepsin. Microsomes were incubated in the absence (1, 2) or presence (3, 4) of proteinase K and in the absence (1, 3) or presence (2, 4) of Triton X-100 followed by SDS-PAGE and immunoblot analysis (see Section 2 for details).

3.8. Membrane topology

To establish hepsin topology in the microsomal membrane, microsomal vesicles were treated with proteinase K in the absence and presence of detergent followed by SDS-PAGE and Western blot (Fig. 6). The immunoreactive band corresponding to hepsin precursor disappears in the presence, but not absence, of detergent showing that hepsin C-terminus faces the lumen of the endoplasmic reticulum.

4. Discussion

Although hepsin was originally identified as long ago as 1988 [13] and subsequent studies demonstrated its importance for cell physiology [15,16], the pure enzyme has not been available for enzymatic and molecular characterization so far. The finding

that hepsin is present in the microsomal membranes has allowed this fraction to be used as the starting material for purification. The use of detergent was necessary for the solubilization of the enzyme from the microsomal membrane and for keeping it soluble at subsequent purification steps. These results are consistent with the hydrophobic association of hepsin with the membrane, probably through a hydrophobic anchor at the N-terminus [13]. Treatment with proteinase K demonstrated that the C-terminal part of the molecule which belongs to the catalytic subunit faces the lumen of the endoplasmic reticulum, a topology characteristic of type II integral membrane proteins. This would result in the extracellular orientation of the catalytic subunit upon transportation to the plasma membrane.

Although it is only the 50 kDa single-chain hepsin precursor that is detectable in the microsomal membranes, Boc-Gln-Arg-Arg-NH-Mec is still cleaved by this fraction, which can be due to the presence of either trace amounts of mature hepsin that escape immunodetection or proteinases other than hepsin with a similar substrate specificity. Extraction of the microsomal membranes with sodium cholate triggers the conversion of hepsin precursor to the active form, which is manifested both in increased total activity and the appearance of a 32 kDa band on SDS-PAGE under reducing conditions. Apparently, extraction removes some inhibitory factor(s) since we were unable to observe any conversion even after prolonged incubation of microsomal membranes at 37°C (data not shown), whereas after solubilization and during further purification gradual maturation took place even in fractions stored at 4°C. It remains to be established whether activation is autocatalytic or catalyzed by another microsomal proteinase. A similar activation phenomenon was observed by others during the purification of membrane-bound trypsin-like proteinases from adrenal chromaffin granules [25], liver microsomes [10], and intestinal mucosa [12].

Three chromatographic steps have proved to be sufficient to obtain electrophoretically homogenous hepsin, with benzamidine-Sepharose chromatography being by far the most efficient one. The molecular weight of pure hepsin on non-reducing SDS-PAGE is ca. 50 kDa, which is higher than the value of 45 kDa predicted on the basis of the cDNA sequence [17]. It is unclear whether this is due to peculiarities in the electrophoretic mobility of hepsin or to some post-translational modifications. Rat hepsin contains a potential N-linked carbohydrate attachment site at Asn-111 [17], and glycosylation could result in a somewhat higher molecular mass than predicted.

Comparing hepsin activities towards a series of fluorogenic peptides demonstrates the enzyme to be an arginine-specific proteinase. Its rather high selectivity, which is evident from the marked effects of amino acids in the P_2 and P_3 positions on cleavage efficiency, suggests that it might be a kind of a processing proteinase. In line with this view is the recent evidence that hepsin expressed on the cell surface of baby hamster kidney cells specifically activates human factor VII [16].

Inhibition pattern of hepsin is typical of trypsin-like serine proteinases. In addition, thiol-directed agents cause partial inhibition. The effect of the thiol-reducing agent dithiotreitol may be due to its disrupting one of the several disulfide bonds maintaining the tertiary structure of all trypsin-like proteinases, while the effect of thiol oxidizing agents E-64 and *p*-chloromercuribenzoate points to the presence of some critical free SH-group(s) in the enzyme.

By combination of its enzymatic and molecular properties hepsin is very similar to at least two other microsomal trypsin-like proteinases isolated so far. An enzyme from porcine intestine mucosa [12] exhibits the same substrate specificity, pH-dependence and inhibitor sensitivity (except that it is not sensitive to thiol-directed agents) and has a molecular mass of 50 and 32 kDa on non-reducing and reducing SDS-PAGE, respectively. However, no band which would correspond to a smaller subunit was identified, and the enzyme was suggested to be a homodimer. By its partial amino-acid sequence this proteinase is also clearly different from hepsin. Another enzyme has been isolated from rat liver microsomes by a procedure very similar to that used in the present study [10]. Again, in its specificity, pH-dependence and inhibition profile (except rather low sensitivity to $PhMeSO_2F$) it is very close to hepsin, and its molecular weight on reducing SDS-PAGE is 32 kDa. However, the molecular weight under non-reducing conditions is the same meaning that this proteinase does not have the subunit structure characteristic of hepsin. Earlier we have isolated two microsomal membrane-bound serine proteinases using the sequence of the

same chromatographic steps, but based on a different assay, the degradation of microsomal cytochrome *P*-450 2E1 [26]. The proteins had a molecular weight of 32 kDa and showed an inhibition profile of serine proteinases. Several considerations, however, argue against either of the cytochrome *P*-450-degrading proteinases being identical to hepsin. First, DEAE-Sepharose chromatography clearly resolved the peaks of the cytochrome *P*-450-degrading activities from that of a peptidase cleaving on the carboxy side of the Arg residue [26], whereas hepsin immunoreactivity peaks were shown in the present study to always coincide with the latter. Second, cytochrome *P*-450 proteinases were optimally active at pH 8 [26], while hepsin activity increases continuously at least up to pH 9. Third, the sequencing of peptides obtained after cytochrome *P*-450 digestion showed that the cytochrome *P*-450 proteinases cleave preferably after Lys (Hellman, U., Zhukov, A., and Ingelman-Sundberg, M., unpublished data), whereas hepsin shows a strong preference for Arg (Table 2). Finally, no small subunit was detected in cytochrome *P*-450 proteinases. The final answer about the relationship between the cytochrome *P*-450 proteinases and hepsin will be possible to give after the former have been sequenced.

One cannot completely rule out the possibility that some of the hepsin found in the microsomal fraction is due to contamination with plasma membranes. For accurate quantitative estimates to be obtained, hepsin contents in the microsomal and plasma membrane fractions should be compared and backed by data on marker enzyme activities. Still the presence of certain amounts of hepsin precursor in microsomes is inevitable, since all plasma membrane proteins are synthesized and therefore transiently present in the endoplasmic reticulum. Important is whether hepsin precursor can be converted to the active form and play any physiological role in the endoplasmic reticulum or at some more distal position along the secretory pathway. The fact that only the 50 kDa precursor can be detected in microsomes seems to speak against this possibility. It should be borne in mind, however, that even in the plasma membrane only the precursor, but not the mature form, was detected in the earlier studies [14]. Generally speaking, the presence of an enzyme on the cell surface does not always mean that this is the only or even the main site for it to exert its physiological activity. Thus cytochrome *P*-450, a resident protein of the endoplasmic reticulum, has also been shown to follow a vesicular route to the plasma membrane and be present on the cell surface in a functionally active form [27,28]. The absence of mature hepsin in microsomes could be explained if one assumes that activation does take place in the endoplasmic reticulum, but only under yet unknown physiological conditions. Finally, it might be worth reminding that the two above mentioned serine proteinases [10,12] which are very similar to hepsin in many aspects and therefore are likely to have a similar function were also isolated from the microsomal fraction. Therefore, the question of the possible physiological activity of hepsin inside the cell remains open until the activation mechanism of hepsin precursor has been elucidated.

As pointed out above, hepsin might be involved in the proteolytic modification of newly synthesized proteins. Alternatively, endoplasmic reticulum is known to contain a proteolytic quality control system eliminating those of the newly synthesized proteins which are unassembled or misfolded [29]. Little is known about the identity of the enzymes involved, but certain evidence is consistent with their serine proteinase nature [30] thus offering hepsin another possible role in cell physiology.

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