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Purification and Characterization of Bleomycin Hydrolase, Which Represents a New Family of Cysteine Proteases, from Rat Skin

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Bleomycin (BLM) hydrolase, which hydrolyzes the carboxyamide bond in the β-aminoalanine moiety, was purified from newborn rat skin. The enzyme was purified 2,500-fold over the crude extract to apparent homogeneity in five steps in the presence of 2-mercaptoethanol: 45-55% ammonium sulfate fractionation, followed by chromatographies on Sephacryl S-200, DEAE-cellulofine, Phe-Superose, and Mono Q ion-exchange. The native enzyme had a molecular mass of 280 kDa according to gel filtration. The subunit molecular mass was estimated as 48 kDa by SDS-PAGE, indicating that the enzyme was comprised of six identical subunits. The amino acid sequence of its NH_2-terminus was determined to be acetyl-Met-Asn-Asn-Ala-Gly-Leu-Asn-Ser-Glu-Lys-, which was not found in the amino acid sequence database. The optimum pH of the enzyme was 7.5 with pepleomycin (PLM). The K_m and V_{max} values were 2.1 mM and 6.8 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ for PLM, and 1.8 mM and 7.2 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ for BLM-A_2, respectively. The enzyme activity was inhibited by iodoacetic acid, N-ethylmaleinimide (NEM), and p-chloromercuribenzoic acid (pCMB) as well as divalent cations such as Cu^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+}. It was effectively inhibited by a cysteine protease inhibitor E-64. However, cystatins A and C did not inhibit the activity. BLM hydrolase exhibited broad aminopeptidase substrate specificity towards aminoacyl-β-naphthylamides such as basic, neutral, and hydrophobic amino acid residues, as well as acidic residues. These results indicated that BLM hydrolase represents a new family of cysteine proteases. Western blotting and immunohistochemical analyses showed that BLM hydrolase is ubiquitous in various rat tissues but at low levels in lung and adult skin tissues, suggesting that this enzyme plays an important role in the metabolism of antibiotics.

Key words: antibiotics, bleomycin, bleomycin hydrolase, cysteine protease, rat skin.

The bleomycins (BLMs) are a group of antitumor antibiotics that are isolated from the organism *Streptomyces verticillis* and have been used extensively to treat many human malignant cancers (*1-3*). The proposed *in vivo* mechanism of action of these compounds involves both single- and double-strand DNA scission (*4, 5*). A unique feature of BLMs is the lack of significant bone marrow, hepatic, or renal toxicity (*3*). However, because of some increased resistance to the therapeutic effect of BLMs, several investigators have attempted to isolate biochemical factors responsible for altering cellular sensitivity to these agents (*6, 7*). One such factor, isolated from rabbit lung as the enzyme responsible for inactivation of BLM, is BLM hydrolase (*8-11*). BLM hydrolase hydrolyzes the carboxyamide bond of the β-aminoalanine moiety on the BLM molecule to a carboxylic acid (*6*). The susceptibility of both normal and malignant tissues to BLM-induced toxicity depends on the level of enzyme activity of BLM hydrolase (*12-14*). Some BLM-resistant cells contain higher levels of BLM hydrolase than sensitive cells (*15, 16*). The enzyme is ubiquitous in mammalian tissues, but it is so labile that only that from rabbit has been biochemically characterized. Thus, it should be valuable for BLM chemotherapy to identify and characterize BLM hydrolase from various tissues. So far, there is little information about the nature of BLM hydrolase from skin. Here, we describe the purification and biochemical characterization of BLM hydrolase from rat skin. The results indicated that this enzyme represents a new family of cysteine proteases.

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Abbreviations: APMSF, 4-(amidinophenyl)-methanesulfonylfluoride; BANA, N-α-benzyl-arginine-β-naphthylamide; BLM, bleomycin; CBz, benzyloxycarbonyl; E-64, 1-(transeposuccinyl-1-leucylamide)-4-guanidinobutane; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; MCA, 4-methyl-coumaryl-7-amide; NEM, N-ethylmaleinimide; PBS, phosphate-buffered saline; pCBM, p-chloromercuribenzoic acid; pNA, 4-nitroanilide; PLM, pepleomycin; PMSF, phenylmethylsulfonyl fluoride; Pyr, L-pyroglutamic acid; Suc, succinyl.

MATERIALS AND METHODS

Materials—BLM and pepleomycin (PLM) for clinical use were provided by Nippon Kayaku (Tokyo). Main compo-

nent BLM-A_2 was isolated by fast protein liquid chromatography (FPLC) on a Mono S column. Chromogenic peptide substrates and soybean trypsin inhibitor were obtained from Sigma (St. Louis, USA). Glu-γ-β-naphthylamide (Glu-γ-β-NA) was from BACHEM Bioscience (Switzerland). Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Pyr-Phe-Leu-pNA were from Peptide Institute (Osaka). The protease inhibitor set including antipain, aprotinin, bestatin, chymostatin, 1-(transepoxysuccinyl-1-leucylamide)-4-guanidinobutane (E-64), leupeptin, pepstatin, 4-(amidinophenyl)-methanesulfonylfluoride (APMSF), and phosphoramidon was from Boehringer Mannheim Biochemica (Germany). Dithiothreitol (DTT), *o*-phenanthroline, *p*-chloromercuribenzoic acid (pCMB), and phenylmethylsulfonyl fluoride (PMSF) were from Wako Pure Chemicals (Tokyo). Sephacryl S-200 and FPLC Mono S, Mono P and Phe-Superose were purchased from Pharmacia-LKB (Sweden). DEAE-cellulofine was from Seikagaku Kougyou (Tokyo). Cystatins A and C were purified from human skin (*17*) and urine (*18*), respectively. All other reagents used were of analytical grade.

Enzyme Assays—The BLM hydrolase activity was measured by means of high performance liquid chromatography (HPLC) (*8*). The reaction mixture was composed of 30 μl of 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT, 10 μl of enzyme solution, and 20 μl of metal-free BLM-A_2 or PLM (4.8 mg/ml) and was incubated for 2 h at 37°C. The reaction was terminated by adding 180 μl of ice-cold methanol and 10 μl of 10 mM $CuSO_4$ on ice. The mixture was centrifuged at 12,000 $\times g$ for 10 min. The supernatant (20-100 μl) was injected into a CAPCELL PAK C8 SG300 column (4.6 $\times$ 150 mm, Shiseido, Tokyo), which was then eluted with a mobile phase consisting of methanol/acetonitrile/20% ammonium acetate/acetic acid (560 : 440 : 100 : 0.5). Two peaks were eluted. The faster peak was deamide-BLM-A_2 or deamide-PLM and the latter one was BLM-A_2 or PLM. BLM-A_2 and deamide-BLM-A_2 were detected by fluorescence as described by Lazo and Humphreys (*13*). PLM and deamide-PLM were measured by UV absorbance at 290 nm (*9*). One unit of BLM hydrolase activity hydrolyzes 1 μg of BLM-A_2 or PLM per min at 37°C.

We measured the aminopeptidase activity of BLM hydrolase towards various aminoacyl-β-NA substrates. The activity was routinely measured with Arg-β-NA. After incubation for 5 min in 1 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM DTT and 5 mM EDTA at 37°C, the enzyme reaction was started by adding of 20 μl of 20 mM Arg-β-NA and continued at 37°C for 10-30 min. The reaction was stopped with 1 ml of 25% acetic acid in ethanol (v/v) and 1 ml of 0.2% *p*-dimethylaminocinamaldehyde (w/v). The color was developed at 37°C for 15 min, then measured at 545 nm.

Enzyme Purification—BLM hydrolase was purified from an epidermal homogenate of newborn rat skin (2-day-old, Sprague-Dawley rat). The epidermis (25 g of wet weight) was homogenized with ten volumes of 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl, 10 mM 2-mercaptoethanol, and 5 mM EDTA (buffer A) in a Polytron, followed by a glass homogenizer. After centrifugation of the homogenate at 12,000 $\times g$ for 20 min, the supernatant was fractionated with 45-55% ammonium sulfate saturation, then the precipitate was dialyzed against buffer A without EDTA. The dialyzate was centrifuged at 12,000 $\times g$ for 20 min to remove insoluble materials. The supernatant was applied onto a Sephacryl S-200 column and eluted with buffer A. Fractions with both BLM hydrolase and aminopeptidase activities were collected (Fig. 1A) and concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrate was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 mM 2-mercaptoethanol (buffer B), then applied onto a DEAE-cellulofine column (2.5 $\times$ 30 cm) equilibrated with buffer B. After the unadsorbed proteins had been completely removed from the column with buffer B, the adsorbed protein was eluted with a linear gradient of NaCl from 0 to 0.35 M in buffer B. The active fractions were pooled and dialyzed against the buffer B containing 1.5 M $(NH_4)_2SO_4$. The dialyzate was applied to a Phe-Superose column and the enzyme was eluted by lowering the $(NH_4)_2SO_4$ concentration. The active fractions were dialyzed against buffer B and the dialyzate was applied onto a Mono Q column equilibrated with buffer B. The protein was eluted with a linear gradient of 0 to 0.35 M NaCl in buffer B (Fig. 1B). The active fractions were collected and used as the purified BLM hydrolase.

Protein Determination—The protein concentrations were determined by the method of Lowry *et al.* (*19*) using bovine serum albumin as a standard.

Determination of Optimum pH—The optimum pH of BLM hydrolase activity was determined using PLM as the substrate in buffers at various pHs. The buffers were all 50 mM sodium salts or Tris-HCl: acetate for pH 4 to 6, phosphate for pH 6 to 7, Tris-HCl for pH 7 to 9, and carbonate for above pH 9.

Kinetic Studies—The purified enzyme was incubated with various concentrations of BLM-A_2 or PLM for 2 h at 37°C. The samples were then assayed for BLM hydrolase activity as described above and the kinetic parameters K_m and V_{max} were determined by Lineweaver-Burk analysis.

Inhibition Studies—The purified BLM hydrolase was preincubated with various concentrations of the putative inhibitors, as specified, for 15 min at room temperature. The samples were then incubated with PLM for 2 h at 37°C and assayed for BLM hydrolase activity by HPLC as described above.

Molecular Mass Determination—Gel filtration was performed on a Sephacryl S-200 column (3.5 $\times$ 95 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1 mM 2-mercaptoethanol. The void volume was determined with blue dextran. Horse ferritin (440 kDa), bovine catalase (240 kDa), rabbit aldolase (160 kDa), and bovine serum albumin (67 kDa) were used as the standard proteins.

Chromatofocusing—The final preparation was applied to a chromatofocusing Mono P column equilibrated with 10-fold diluted Polybuffer 94 adjusted to pH with 25 mM imidazole hydrochloride (pH 7.4). The column was eluted with a linear pH gradient of Polybuffer 74 (pH 4.0). The eluates were immediately neutralized with 0.3 M sodium phosphate buffer (pH 7.4). BLM hydrolase activity was determined as described above.

Preparation of Antibody against BLM Hydrolase—Antiserum against BLM hydrolase was raised in two rabbits. The IgG fraction from the antiserum was isolated by fractionation with ammonium sulfate and purified by affinity-chromatography on Protein G-Sepharose and enzyme-Sepharose 4B columns.

Electrophoresis and Western Blot Analysis—Native PAGE was carried out with a minislab gel apparatus using a 7.5% gel at discontinuous pH (*20*). Aminopeptidase activity staining in nondenaturing gels was performed according to the method of Hirsch *et al.* (*21*) using Leu-β-NA as substrate. SDS-PAGE was performed according to Laemmli (*22*), using a 10% gel. The proteins were transferred from the gel to a P-membrane sheet (ATTO, Tokyo) by horizontal electrophoresis. Proteins were immunostained with rabbit anti-BLM hydrolase IgG by means of the Promega protocol using alkaline phosphatase conjugated goat anti-rabbit antibody (Promega Biotec, USA). Proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass of the enzyme was determined using a standard kit (BioRad, USA).

Analyses of the Amino Acid Composition and Sequence—The amino acid composition of the enzyme was determined using a Hitachi 5200 amino acid analyzer after hydrolysis with 6 M HCl in a sealed evacuated tube at 110°C for 24 h. Cysteine and tryptophan residues were also analyzed after hydrolysis with 4-methansulfonic acid according to the method of Simpson *et al.* (*23*). The purified enzyme was modified with 4-vinylpyridine after reduction with tri-*n*-butylphosphine in 0.5 M Tris-HCl buffer (pH 7.5) containing 7 M guanidinehydrochloride and 10 mM EDTA (*24*). The reaction solution was exhaustively dialyzed against distilled water. The lyophilized sample was subjected to cleavage by TPCK-treated trypsin in 0.1 M ammonium acetate (pH 8.0). The digested peptides were treated with a Protein N-terminal Deblocking kit (Takara) according to the method of Tsunasawa *et al.* (*25*). The NH_2-terminal amino acids were determined using an Applied Biosystem 470A Automated Protein Sequencer (*26*). An acetylamino acid was identified by reversed phase HPLC according to the method described previously (*27*).

Circular Dichroism (CD)—CD spectra were measured at 25°C under constant nitrogen flush on a JASCO J-600 recording dichrograph. Cells of 2 or 1 mm in length were used, depending upon the protein concentration. The CD data are expressed as mean residue ellipticity, $[\theta]$, in which the mean residue molecular weight of the enzyme was calculated as 105 from the molecular mass and amino acid composition.

Immunohistochemistry—Tissues were taken from newborn and adult rats and fixed for 3 h in 4% neutral paraformaldehyde at room temperature followed by embedding paraffin. Serial paraffin sections (6 μ) were placed on glass slides and stained with hematoxylin and eosin. For immunohistochemical examination, sections were deparaffinized, rehydrated, and then incubated at room temperature for 20 min with 5% normal swine serum. After washing three times in phosphate-buffered saline (PBS), they were incubated for 1 h at room temperature with rabbit anti-enzyme IgG and washed three times in PBS. The sections were incubated with peroxidase-conjugated swine anti-rabbit IgG(H+L). After washing, they were developed with 0.04% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-HCl (pH 7.5) at room temperature, dehydrated, clarified, and mounted. Control sections were incubated with preimmune serum.

RESULTS

Purification and Some Biochemical Characterization of BLM Hydrolase from Rat Skin—BLM hydrolase was purified from newborn rat epidermis as summarized in Table I. The enzyme activity was detectable in epidermal homogenate in the presence of 10 mM 2-mercaptoethanol and 5 mM EDTA. Other sulfhydryl compounds had a similar effect upon the BLM hydrolase activity. Both BLM hydrolase and aminopeptidase activities coeluted throughout the process of purification (Fig. 1, A and B), although BLM hydrolase was separated from another aminopeptidase activity with low molecular mass, which was identified as cathepsin H, by Sephacryl S-200 gel filtration. The results indicated that the two activities originated from the same enzyme. A purification of about 2,500-fold with a yield of 13% was achieved. The purified enzyme was resolved as a single symmetrical peak with a molecular mass of about 280 kDa by gel filtration (Fig. 2) as well as a band which demonstrated the activity staining by native PAGE (Fig. 3, A and B). SDS-PAGE of the enzyme revealed a single band with a molecular mass of about 48 kDa (Fig. 3C). These results suggested that BLM hydrolase is a hexameric enzyme. The isoelectric point of pH 5.6 for the enzyme was estimated by chromatofocusing using a Mono P column (data not shown). Table II shows that BLM hydrolase is composed of about 435 amino acid residues. The enzyme uniformly contained all of the amino acids with small amounts of Cys and Trp. A comparison of the amino acid composition between rat and rabbit (*10*) enzymes revealed a significant difference in the amounts of Asx, Ser, Gly, and Val. Direct sequencing of BLM hydrolase failed due to a blocked amino terminus. The amino acid sequence of NH_2-terminus was determined to be acetyl-Met-Asn-Asn-Ala-Gly-Leu-Asn-Ser-Glu-Lys-. The NH_2-terminal sequence of rat BLM hydrolase showed no homology to those of other cysteine proteases listed in the database. A CD spectrum of BLM hydrolase in the far ultraviolet region revealed two negative bands at 233 and 210 nm with $[\theta]$s of $-14{,}000$ and $-11{,}000$, respectively, indicating the presence of α-helix and β-strand structures in the protein

TABLE I. **Summary of the purification of BLM hydrolase from rat skin.** For details, see "MATERIALS AND METHODS." Data are expressed as the average of three purification procedures.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Yield (%)
Crude extract	3,605[a]	7,210	0.5	—	100
45-55% $(NH_4)_2SO_4$	1,460	164	8.9	18	41
Sephacryl S-200	1,018	48	21.2	42	28
DEAE-Cellulofine	821	12	68.4	137	23
Phe-Superose	640	0.85	752.9	1,506	18
Mono Q	471	0.38	1,239.5	2,479	13

[a]Arbitary value for the high concentration of protein in homogenate.

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Characterization on BLM Hydrolase Activity—Lineweaver-Burk plots revealed a linear relationship between $1/V$ (unit/mg^{-1}) and $1/[S]$ (mM^{-1}), and gave K_m and V_{max} values of 2.1 mM and 6.8 μmol·mg^{-1}·h^{-1} for PLM, and 1.8 mM and 7.2 μmol·mg^{-1}·h^{-1} for BLM-A_2, respectively. These were in agreement with those of BLM hydrolase from rabbit lung and liver (*9, 10*). Aliquots of the purified enzyme were incubated with PLM in the presence of various buffers from pH 4 to 10. The optimal pH of the enzyme activity was around pH 7.5 (Fig. 4). Table III summarizes the effects of various substances on BLM hydrolase activity. The titration curve showed that the most potent inhibitor tested was E-64, which totally inhibited BLM hydrolase activity at concentrations equimolar to those of the enzyme (data not shown). NEM, iodoacetic acid, and pCMB were also potent inhibitors of BLM hydrolase activity. Leupeptin and puromycin at higher concentrations partially inhibited BLM hydrolase activity, but serine protease inhibitors such as APMSF, aprotinin, chymostatin and PMSF, bestatine and pepstatin had little or no effect. Protein cysteine protease inhibitors, cystatins A or C, did not inhibit BLM hydrolase activity. The chelators EDTA, EGTA and phosphoramidon also had no effect, whereas *o*-phenanthroline was inhibitory. This may be the result of contamination of the reagent by metal ions. The divalent metal ions Hg^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} strongly inhibited the enzyme activity, whereas Mn^{2+}, Ca^{2+}, and Mg^{2+} had no effect (Table IV).

We have examined the properties of the enzyme as its aminopeptidase activity towards various aminoacyl-β-NA substrates (Table V). Rat BLM hydrolase released basic, neutral, and hydrophobic amino acid residues as well as acidic residues, indicating a broad aminopeptidase specificity, but it did not release Pro. Met-β-NA was a good substrate for the aminopeptidase function of this enzyme.

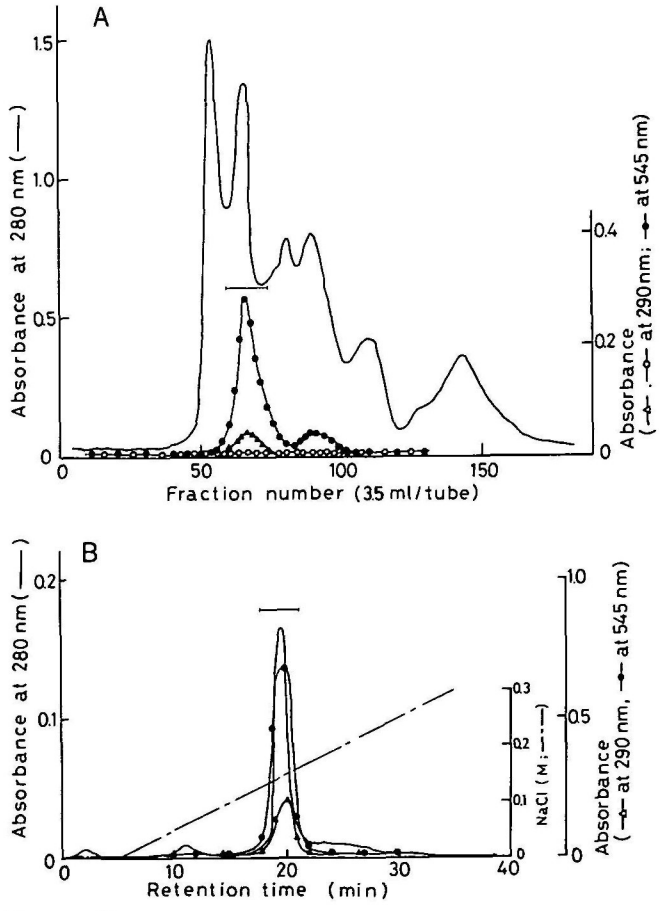

Fig. 1. **Chromatographic purification of rat skin BLM hydrolase.** A: Sephacryl S-200 gel filtration. A dialyzed sample from ammonium sulfate fractionation was applied to a Sephacryl S-200 column (3.5×95 cm) equilibrated with buffer A and eluted with the same buffer. Fraction size, 3.5 ml. The fractions under the bar were pooled. B: Mono Q ion-exchange chromatography. The sample obtained by Phe-Superose column chromatography was applied to an FPLC Mono Q column and eluted with a linear gradient from 0 to 0.3 M NaCl in a total volume of 35 ml at flow rate of 1 ml/min. Fraction size, 1 ml. The fractions under the bar were pooled as the purified enzyme. The activities of BLM hydrolase in the presence (▲) and in the absence (△) of 10 mM DTT and aminopeptidase in the presence of DTT (●) were assayed as described in "MATERIALS AND METHODS."

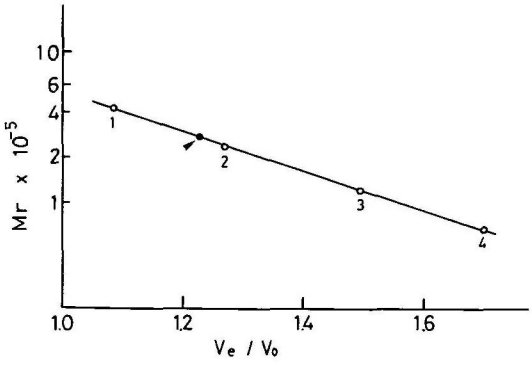

Fig. 2. **Determination of molecular mass of rat BLM hydrolase by Sephacryl S-200 column chromatography.** The column was 3.5×95 cm. The buffer was 25 mM Tris-HCl (pH 7.5) containing 1 mM 2-mercaptoethanol and 0.15 M NaCl. A semilog plot of ($M_r \times 10^{-5}$) *vs.* V_e/V_0 for BLM hydrolase (arrow head) and standard proteins (1, horse ferritin, M_r=440 kDa; 2, bovine catalase, M_r=240 kDa; 3, rabbit aldolase, M_r=160 kDa; 4, bovine serum albumin, M_r=67 kDa) is shown.

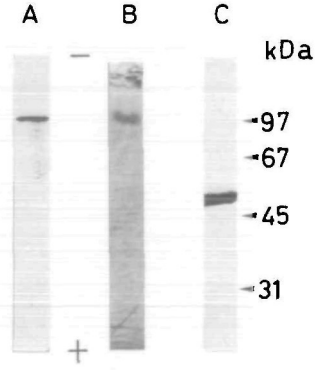

Fig. 3. **Gel electrophoresis of BLM hydrolase.** A, native PAGE using a 7.5% gel; B, aminopeptidase activity staining in native PAGE gel; C, SDS-PAGE using a 10% gel. The sample from the FPLC Mono Q column was analyzed. Electrophoresis and aminopeptidase activity staining were carried out under the conditions described in "MATERIALS AND METHODS." Positions of molecular mass markers are shown in kDa.

TABLE II. **Amino acid composition of BLM hydrolase from rat skin.** The values are given as the number of residues per subunit. *Values in parentheses are the nearest integers.*

Amino acid	Rat	Rabbit[a]
Asx	57.4 (57)	34
Thr	24.3 (24)	23
Ser	17.9 (18)	75
Glx	48.6 (49)	54
Pro	17.1 (17)	27
Gly	25.8 (26)	63
Ala	21.1 (21)	29
Cys	7.3 (7)	—
Val	40.9 (41)	29
Met	12.3 (12)	12
Ile	15.5 (16)	15
Leu	34.2 (34)	32
Tyr	13.4 (13)	14
Phe	26.5 (27)	19
Lys	33.8 (34)	39
His	15.3 (15)	15
Arg	16.2 (16)	20
Trp	7.9 (8)	—
Total	(435)	(500)

[a]Summarized from the data presented in Ref. *11*.

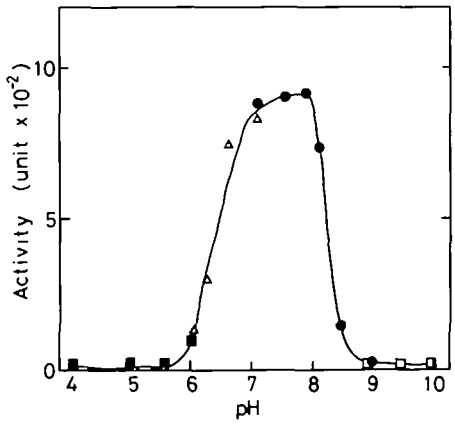

Fig. 4. **The pH dependence of the BLM hydrolase activity.** Activity towards PLM was measured as described in "MATERALS AND METHODS," in the following buffers: ■, acetate; △, phosphate; ●, Tris-HCl; □, carbonate.

TABLE III. **Effects of various substances on BLM hydrolase activity.** The purified enzyme (0.1 unit) was preincubated for 15 min at room temperature in 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT with one of the substances at the final concentration indicated. The activities were assayed as described in "MATERIALS AND METHODS." The activities are expressed as the percent of those without the substances in preincubation.

Substances	Concentration	Relative activity (%)
None		100
Antipain	100 μM	102
APMSF	80 μM	98
Aprotinin	100 μM	94
Bestatin	120 μM	92
Chymostatin	120 μM	98
E-64	160 μM	0
Leupeptin	120 μM	89
Pepstatin	120 μM	92
Phosphramidon	120 μM	102
PMSF	100 μM	92
Puromycin	120 μM	87
NEM	1 mM	22
Iodoacetic acid	1 mM	6
pCMB	0.5 mM	2
EDTA	1 mM	108
EGTA	1 mM	100
o-Phenanthroline	0.5 mM	38
Cystatin A	1 μM	94
Cystatin C	1 μM	100

TABLE IV. **Effects of metal ions on BLM hydrolase activity.** The purified enzyme (0.1 unit) was preincubated for 15 min at room temperature in 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT with one of the metal ions at the final concentration indicated. The activities were assayed as described in "MATERIALS AND METHODS." The activities are expressed as the percent of those without the metal ions in preincubation.

Metal ions	Concentration (mM)	Relative activity (%)
None		100
Mn^{2+}	1	98
Cu^{2+}	1	12
Zn^{2+}	1	38
Cd^{2+}	0.5	26
Hg^{2+}	0.5	0
Ca^{2+}	1	92
Mg^{2+}	1	104

BLM hydrolase weakly hydrolyzed BANA, but not other substrates with substituted amino groups. No activity was found toward protein substrates. These results revealed that BLM hydrolase is a cysteine protease having cathepsin H-like activity.

Expression of BLM Hydrolase Protein in Rat Tissues— We examined the expression of BLM hydrolase protein in rat tissues by means of Western-blotting and immunoperoxidase staining. Figure 5A shows that a single component with a molecular mass of 48 kDa immunoreacted with rabbit anti-rat BLM hydrolase IgG in various homogenates from adult rat. The band intensity of the enzyme in lung and skeletal muscle homogenates (lanes 4 and 8) was very weak, indicating the presence of less BLM hydrolase protein than in other tissues. In addition, the band in homogenate from adult rat skin (lane 9) was weaker than that in the newborn (lane 1). The enzyme was ubiquitous in human, rat, rabbit, and mouse liver (Fig. 5B). Immunoperoxidase staining shows that BLM hydrolase was present in the epithelia of rat tissues. Newborn and adult rat skin sections were stained in germinative cells of the epidermis as well as in hair follicles as epidermal appendages, where the thin granular cell layer was intensely stained (Fig. 6A). The superepithelial cells in esophagus were intensely stained, whereas the basal cells were negative (Fig. 6B). Thus BLM hydrolase is expressed in differentiating nonproliferative cells *in vivo*.

DISCUSSION

Various cysteine proteases have been isolated from mammalian cells, including the lysosomal cathepsins (B, H, L, and S) (*28, 29*), cytosolic Ca^{2+}-dependent calpains (*30*), and others (*31-33*), most of which have been characterized as endopeptidases. Only cathepsin H reportedly cleaves typical synthetic chromogenic peptide and oligopeptide substrates in the manner of an aminopeptidase (*34*). A few aminopeptidases with cathepsin H-like enzymatic activity from mammalian tissues have been characterized (*35-37*) and are distinguished from cathepsin H by molecular mass,

pI, and pH optimum. The best studied aminopeptidase is BLM hydrolase from rabbit (*8-11, 36*).

Here, we purified to homogeneity and characterized BLM hydrolase from newborn rat skin. BLM hydrolase was separated from cathepsin H by Sephacryl S-200 gel filtration (Fig. 1A). The activity was stabilized by adding sulfhydryl compounds through the process of preparation. In addition, the optimal activity of BLM hydrolase depended upon the concentration of sulfhydryl compounds at neutral pH. These findings suggested that a cysteine moiety of the enzyme is essential for expression of its activity. We confirmed that sulfhydryl reagents such as NEM, iodoacetic acid, or pCMB, as well as divalent heavy metal ions inhibited the enzyme activity (Tables III and IV). The cysteine protease inhibitor E-64 was the most effective inhibitor of BLM hydrolase. However, BLM hydrolase was not inhibited by cystatin A or C, both of which strongly inhibit cathepsin H (*38*). This is a remarkable feature that distinguished BLM hydrolase from cathepsin H. BLM hydrolase exhibited broad aminopeptidase substrate specificity towards various aminoacyl-β-NAs and a weak activity towards BANA, suggesting that it has a tertiary structure partially similar to cathepsin H. But BLM hydrolase had no endopeptidase activities towards proteins and synthetic peptides with a substituted amino group (Table V). These inhibition profiles and substrate specificity indicated that BLM hydrolase should be classified as

TABLE V **Substrate specificity of BLM hydrolase toward β-naphthylamide derivatives of various amino acids.** The purified BLM hydrolase (0.1 unit) were assayed for its activity towards chromogenic substrates as described in "MATERIALS AND METHODS." Activity towards Arg-β-NA was set at 100% The following substrates were not cleaved: Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Cbz-Leu-β-NA, Cbz-Leu-Leu-Glu-β-NA, Suc-Leu-Leu-Val-Tyr-MCA, Pyr-Phe-Leu-pNA

Amino acid in β-naphthylamide derivatives	Relative activity (%)
Arg	100
Lys	109 2
Glu	60 4
Gly	128 1
Ala	76.3
Val	53 0
Leu	125 8
Ile	44 8
Phe	51 3
Tyr	38 6
Trp	44.5
Ser	150.5
Met	175 4
Pro	0

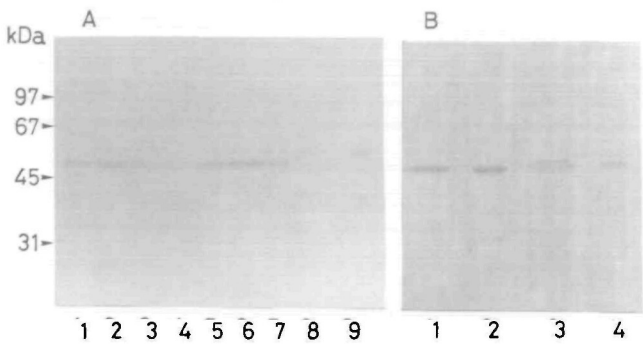

Fig. 5 **Western-blots of BLM hydrolase protein.** A: Extracts (54 μg) of rat tissues (lane 1, newborn skin, lane 2, kidney; lane 3, spleen, lane 4, lung; lane 5, liver; lane 6, stomach and esophagus, lane 7, testis; lane 8, skeletal muscle, lane 9, adult skin) B: Extracts (54 μg) of livers from various species (lane 1, rat; lane 2, human; lane 3, rabbit, lane 4, mouse) The molecular mass was taken from molecular mass standards run on the same blots rabbit phosphorylase *b* (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and bovine carbonic anhydrase (31 kDa).

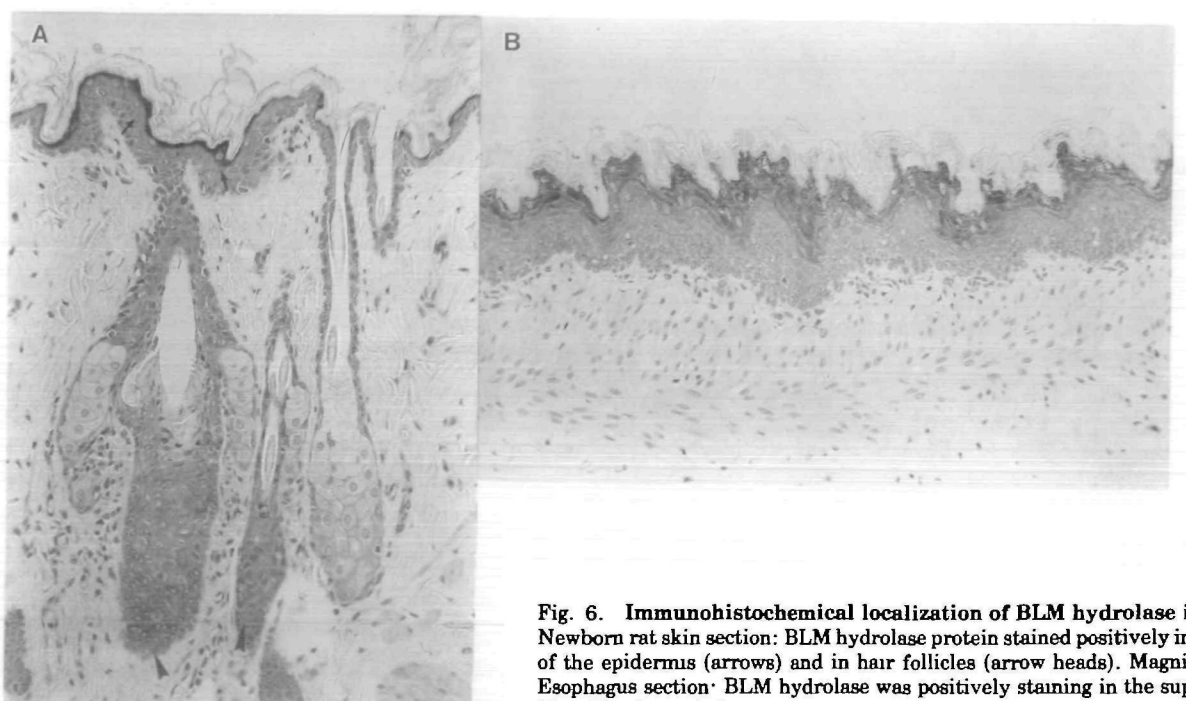

Fig. 6. **Immunohistochemical localization of BLM hydrolase in rat tissues.** A: Newborn rat skin section: BLM hydrolase protein stained positively in germinative cells of the epidermis (arrows) and in hair follicles (arrow heads). Magnification ×240. B: Esophagus section: BLM hydrolase was positively staining in the superepithelial cells. Magnification ×240.

belonging to a new family of cysteine proteases. Rat skin BLM hydrolase is very similar in biochemical properties to the rabbit enzyme (*8-10*). Both BLM hydrolases are multimers of identical subunits with a similar molecular mass, but the enzymes from rabbit lung and liver reportedly have pentameric and hexameric structures, respectively (*9, 10*). According to the analytical data of the enzyme from rat skin, mammalian BLM hydrolase is probably a hexameric enzyme. Rat skin BLM hydrolase was detected as a single fraction by chromatofocusing, although the rabbit lung enzyme was composed of three isomers in a sample at a later step of purification. We do not know yet whether the presence of isomers of rabbit BLM hydrolase resulted from a difference in amino acid composition between the two enzymes (Table II). BLM hydrolase was expressed in various tissues from the rat and in the liver from rat, human, rabbit, and mouse (Fig. 5, A and B). This result is consistent with those of other studies (*11, 39*) that showed the ubiquitous distribution of BLM hydrolase activity among tissues from different species. Western-blotting revealed low levels of BLM hydrolase protein in rat lung and adult rat skin compared with that of other tissues; these tissues are susceptible to BLM toxicity (*13, 40, 41*). This suggested that the amount of BLM hydrolase in normal or tumor tissues is critical in determining the susceptibility to BLM-induced toxicity. Also, the different band intensity of the enzyme in adult and newborn rat skin on Western-blotting may reflect differences in a series of biochemical changes occurring in the differentiation of both epidermal keratinocytes. The lack of BLM hydrolase protein in basal cells in the epidermis and tissues containing epithelia suggests that expression of the enzyme is linked to differentiation *in vivo*.

Many hydrolytic enzymes are present in skin and are thought to participate in the regulation of epidermal cell function (*42*). BLM hydrolase has physicochemical properties and aminopeptidase substrate specificity similar to those of a high molecular mass cysteine protease that has been purified from newborn rat epidermis (*35*). However, the possible relationship between BLM hydrolase and high molecular mass cysteine protease will require further investigation. Cloning and sequencing of a cDNA that encodes rat BLM hydrolase protein should allow precise identification and detailed characterization of this enzyme. Molecular studies along these lines are in progress.

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