J. Biochem. **141**, 69–76 (2007)
doi:10.1093/jb/mvm005

Quantification of Neutral Cysteine Protease Bleomycin Hydrolase and its Localization in Rat Tissues

Yayoi Kamata[1], Yoshiko Itoh[2], Akane Kajiya[1], Sachiyo Karasawa[1], Chie Sakatani[1], Susumu Takekoshi[2], R. Yoshiyuki Osamura[2] and Atsushi Takeda[1,*]

[1]Laboratory of Biochemistry, Faculty of Arts and Sciences, Sagami Women's University, Sagamihara, Kanagawa 228-8533; and [2]Department of Pathology, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

Received August 10, 2006; accepted November 13, 2006; published online December 11, 2006

A neutral cysteine protease, bleomycin hydrolase (BH), was found to be present in the range 3.7–131.1 ng per mg of rat tissues by enzyme-lined immunosorbent assay (ELISA). Newborn rat skin contained the highest amount of BH, and relatively high levels of BH were detected in the kidney and liver of 6-week-old male rats. The tissue distribution of BH in female rats was similar to that in male rats. Moreover, BH was detected in the extracts of erythrocytes and leukocyte-rich cells as well as in those of rat hemo-lymphocytic lineage cell lines by Western blotting. The BH level was increased at 6 weeks after birth and then slightly decreased. By immunohistochemistry, BH was localized as granular staining in the distal and proximal tubular cells of the kidney, and it was also detected in hepatocytes of the liver, in the red pulpy region of the spleen and in neurons of the brain. An immunoelectron microscopic study showed that BH-immunoreactivity was essentially located in the cytoplasm and at the outer membrane of the rough endoplasmic reticulum of epithelial cells of the kidney, as well as in that of hepatocytes of the liver. These results suggest that BH may play ubiquitous and unique roles in rat tissues.

Key words: bleomycin hydrolase, cysteine protease, ELISA, immunocytochemistry, rat tissues.

Abbreviations: BH, bleomycin hydrolase; Cit-β-NA, Citrulline-β-naphthylamide; ELISA, enzyme-linked immunosorbent assay.

Bleomycin hydrolase (BH) was first detected in mouse liver based on its ability to inactivate the anti-tumor antibiotic bleomycin (*1*) and was later purified from rabbit lung and liver, chicken skeletal muscle and rat epidermis (*2–5*). Rabbit, rat, human and chicken cDNAs encoding BH have been isolated (*6–10*), and the deduced amino acid sequences have revealed that the enzyme is a neutral cysteine protease of the papain superfamily (*11*). Furthermore, sequence analysis of the promoter revealed that it has characteristic features of a human housekeeping gene (*12*). BH is widely distributed throughout nature, with homologues found in mammals (*1–3, 5–9*), amphibians (*6*), birds (*4, 10*), yeast, (*13–16*) fish (*17*) and bacteria (*18–20*). The expression of BH mRNA in mammalian tissues are ubiquitous, with some differences between the expression patterns shown by Northern blot and reverse transcription polymerase-chain reaction analyses (*6–9*). BH activity was also detected in tissue homogenates from a wide variety of tissues (*1*). Histochemical reports revealed that BH is primarily localized in stratified epithelial cells in rat tissues (*5*), and its expression is specifically decreased in human cancerous skin (*21*). BH has amino-peptidase activity towards aminoacyl-β-naphthylamide derivatives of different amino acids and short peptides (*4–6, 13–15*), and also exhibits intrinsic exo- and endo-peptidase activities (*10, 15, 16, 22*). BH consists of six subunits with a total molecular weight of about 300 kDa and structurally belongs to a family of self-compartmentalizing proteases with similarity to the 20S proteasome (*2–5, 23*). Also, human BH interacts with an ubiquitin-conjugating enzyme 9 as one of partner components (*24*). These results suggest that BH is involved in the processing of some physiologically important proteins. However, the physiological substrates and functions of BH in proteolytic pathways still remain unknown, and there have been no reports of quantitative analyses of BH.

In the present study, we estimated the amounts of BH in extracts from rat tissues at different ages by enzyme-linked immunosorbent assay (ELISA). Also, we examined the expression of BH protein in rat tissues, especially kidney, spleen, liver and brain, by immunohistochemistry and/or immunoelectron microscopy.

MATERIALS AND METHODS

Materials—Citrulline-β-naphthylamide (Cit-β-NA) was purchased from BACHEM Bioscience (Switzerland). N-hydroxysuccinimide biotin and avidin-peroxidase conjugate were obtained from Pierce (Rockford, IL, USA.) and Sigma Chemical Co. (St Louis, MO, USA), respectively.

*To whom correspondence should be addressed. Laboratory of Biochemistry, Faculty of Arts and Sciences, Sagami Women's University, Sagamihara, Kanagawa 228-8533. Tel: +81-42-742-1428, Fax: +81-42-743-4717,
E-mail: takeda.a.ns@star.sagami-wu.ac.jp

O-Phenylenediamine was from Nacalai Tesque (Kyoto, Japan). Rat cathepsins B, H, and L were partially purified from liver lysosomal extract according to the methods of Towatari *et al*. (*25*), Schwartz and Barrett (*26*) and Kirschke *et al*. (*27*), respectively. All other chemicals used in this study were reagent grade.

Animals—Male and female Wistar rats were purchased from Japan CLEA Inc. at the age of 5 weeks (Tokyo, Japan) and were given commercial laboratory chow (solid) and water freely for various periods. For statistical analyses, 5–6 animals of each different age group were studied.

Cells and Cell Culture—Three rat hemo-lymphocytic lineage cell lines (Shay, RBL-2H3 and Y3-ag1.2.3) were supplied by the Japanese Cancer Resources Bank. For each cell line, $6.0–6.5 \times 10^6$ cells were placed in square plastic dishes at a state near confluence and were maintained in Dulbecco's Modified Eagle's Medium (Dainippon Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal calf serum, penicillin (10 IU/ml) and streptomycin (100 μg/ml).

Purification of BH—Rat BH was purified from an epidermal homogenate of newborn rat skin (2-day-old, Sprague–Dawley rat) according to the method described previously (*5*). Briefly, rat epidermis was homogenized with 10 volumes of 20 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 10 mM 2-mercaptoethanol and 5 mM EDTA (buffer A) in a POLYTRON System PT2100 (KINEMATICA AG, Switzerland) followed by a second homogenization in a glass homogenizer. The supernatant was fractionated by precipitation with 45–55% saturated ammonium sulfate. After removal of insoluble materials, the clear solution was applied to a Sephacryl S-200 column (3×90 cm). After dialysis of the active fraction from the column developed with 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM 2-mercaptoethanol (buffer B), the sample was applied to a DEAE-cellulose column (1×30 cm), and the column was washed with buffer B. The proteins were fractionated with a linear gradient of NaCl from 0 to 350 mM in buffer B. The active fractions were pooled, concentrated by ultrafiltration and dialyzed against buffer B. The dialysate was applied to a Mono Q column-equipped HPLC system. The protein was eluted with the same buffer. BH activity was specifically detected by measuring aminopeptidase activity using Cit-β-NA during the course of preparation. The purified BH showed a single band with *M*r 48 kDa on SDS-PAGE.

Production and Preparation of Antibody against BH—Two Japanese white rabbits were subcutaneously immunized with 0.66 mg of BH emulsified in Freund's complete adjuvant (Difco Laboratories). Additional booster injections were administered 3 weeks later and a further three times at 1-week intervals with Freund's incomplete adjuvant. Animals were bled 1 week after the final injection. The final antiserum diluted 12,000-fold was cross-reacted with BH by solid phase ELISA. The IgG fraction from the antiserum was isolated by fractionation with ammonium sulfate and purified on a HiTrap Protein A Hp column (Amersham Pharmacia Biotech, Sweden). The monospecific IgG was purified as follows:

The IgG from the antiserum was isolated by fractionation with ammonium sulfate and purified by affinity chromatography on a BH-Sepharose 4B column prepared by coupling 500 μg of BH to 0.5 g of CNBr-activated Sepharose-4B (Amersham Pharmacia Biotech, Sweden). The IgG fraction eluted from the column with 100 mM glycine-HCl buffer (pH 2.8) was dialyzed against 10 mM phosphate-buffered saline (PBS) and stored at −80°C until use.

Preparation of Samples—Male Wistar rats ($n = 5–6$) at 3 days after birth, or 6, 23 or 103 weeks of age, and female rats at 6 weeks of age, were killed by bleeding. Various tissues were dissected from the rats and stored immediately at −80°C. They were homogenized in four volumes of 20 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 10 mM 2-mercaptoethanol, 5 mM EDTA and 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride in a POLYTRON homogenizer. The homogenate was centrifuged at 12,000*g* for 30 min followed at 105,000*g* for 1 h at 4°C. The extracts were used for the ELISA assay. Rat blood was obtained by heart puncture in a syringe containing heparin and mixed with an equal volume of 3% dextran T-500 (Pharmacia Ltd., Sweden). It was kept at room temperature for 1 h and then the plasma fraction was collected into a tube. The leukocyte-rich cells were harvested by centrifugation at 500*g* for 5 min and then washed two times with physiological saline containing heparin. The harvested cells were suspended in 0.5 ml of buffer A and sonicated with weak power. Erythrocytes were collected using a syringe and washed with physiological saline containing heparin. They were diluted with an equal volume of distilled water. Both the erythrocyte and leukocyte-rich cell extracts were centrifuged at 12,000*g* for 20 min and then the supernatants were collected as samples. Protein concentrations were determined by the method of Lowry *et al*. (*28*) with bovine serum albumin as a standard.

ELISA—Wells (96 wells/flat bottom) of microtiter plates (IWAKI, Tokyo, Japan) were coated with 100 μl of anti-BH IgG (12 μg/ml) in coating buffer, pH 9.6. The plates were sealed and left overnight at 4°C. After removal of the IgG solution, the wells were blocked with 5% BSA in PBS for 1 h at 37°C and washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). The plates were incubated with 100 μl of samples or standard amounts of purified BH (10–200 ng/ml) for 1 h at 37°C. The wells were washed three times with PBS-Tween and 100 μl of biotin-conjugated IgG (1 μg/ml) diluted in PBS-Tween containing 0.1% BSA was added to each well. The plate was incubated for 1 h at 37°C. After removal of the conjugate and washing three times with PBS-Tween, 100 μl of avidin-conjugated horseradish peroxidase diluted in PBS-Tween containing 0.1% BSA was added to each well. After washing, 100 μl of *O*-phenylenediamine (3 mg/ml) containing 1.82 mM hydrogen peroxide in 0.1 M citrate-phosphate buffer (pH 5.0) was quickly added to the wells in the dark. After 10 min, the reaction was stopped by the addition of 25 μl of 10 M sulphuric acid. The absorbance of each well was recorded at 492 nm in an automated ELISA reader (BIO-RAD Ltd. Tokyo, Japan). The amount of BH was expressed as ng per mg of tissue weight.

SDS-PAGE and Western Blotting—SDS-PAGE was performed according to Laemmli (*29*) using 12.5% or 5–20% gels. The proteins were transferred from the gel to

a P-membrane sheet (ATTO, Tokyo, Japan) by horizontal electrophoresis and then were immunostained with rabbit anti-BH IgG according to the Promega protocol using alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega Biotec., USA).

Immunohistochemistry—Tissues were obtained from adult rats and fixed for 6 h in 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) at 4°C and then embedded in paraffin. Serial paraffin sections (4 μm) 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 goat serum. The brain sections were pretreated with trypsin before the incubation with 5% normal goat serum. After the sections were washed three times in 10 mM PBS, they were incubated for 1 h at room temperature with rabbit anti-BH IgG (diluted 1 : 200) and washed 3 times in PBS. The sections were incubated with peroxidase-conjugated goat anti-rabbit IgG(H + L) (diluted 1 : 40; Dako, Glostrup, Denmark). After washing, the sections were treated with 0.02% 3, 3′-diamino -benzidine (DAB) and 0.006% 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.

Immunoelectron Microscopy—Animals were perfused with PBS containing 4% paraformaldehyde solution under ether anesthesia. After perfusion, the kidneys and livers were harvested and fixed in 4% paraformaldehyde overnight at 4°C. The frozen sections were washed twice in PBS buffer for 10 min, and washed 5 times with 10% sucrose in PBS. The sections were then incubated with rabbit anti-rat BH IgG diluted 1 : 200 in 1% BSA-PBS overnight at 4°C. The sections were then washed 10 times with 10% in sucrose in PBS for 10 min. The sections were then incubated with goat anti-rabbit IgG conjugated with HRP (diluted 1 : 400, Amersham Bioscinece Co., Piscataway, NJ, USA) overnight at 4°C. After incubation with the second antibody, the sections were washed as above and then immersed in 0.1% glutaraldehyde for 5 min. The sections were washed again in PBS for 15 min and incubated with 1% gelatin in 100 mM phosphate buffer. After the sections were washed 5 times with PBS, they were immersed in 0.02% DAB without H_2O_2 for 30 min. The sections were then immersed in 0.02% DAB solution containing 0.006% H_2O_2 for 5 min and placed in a 2% osmium tetraoxide solution in 100 mM phosphate buffer (pH 7.4) for 90 min. They were then washed in buffer for 10 min and dehydrated in a graded series of alcohol (50–100%), after which they were embedded in Epon-filled Beem capsules. Ultrathin sections were collected from the surface of the plastic-embedded tissues and examined with an electron microscope (JEM-1200EX, JOELLTD., Tokyo, Japan).

Subcellular Fractionation—Liver and kidney tissues were homogenized in 10 volumes of isotonic buffer by addition of sucrose (0.25 M final concentration) in a Teflon homogenizer. Subcellular fractionation was accomplished by sequential differential centrifugation to yield pellets of non-disrupted tissues and cellular nuclei (1000*g* for 10 min) and mitochondria (10,000*g* for 20 min). The mitochondrial supernatant was centrifuged at 100,000*g* for 60 min using a RP65T rotor in a Hitachi 65P-7 ultracentrifuge to yield a microsomal pellet containing ribosome and cytosolic supernatant fractions. Each subcellular fraction was identified according to the distribution of the activity of marker enzymes, succinate dehydrogenase, (*30*) glucose-6-phosphatase (*31*), and lactate dehydrogenase (*32*) for mitochondrial, microsomal and cytosolic supernatant fractions, respectively. The purity of each fraction was estimated as the recovery of the sum of the specific activities in the mitochondrial, microsomal and cytosolic fractions.

RESULTS AND DISCUSSION

Western Blotting and Quantification by ELISA of BH in Rat Tissues—We prepared rabbit antiserum against rat BH, and examined its specificity for rat BH by Western blotting. Little cross-reactivity of the antiserum for cysteine proteinases such as cathepsins B, H, and L, and papain as well as other cytosolic proteins was detected on the membrane by staining with the alkaline phosphatase system (Fig. 1). To examine in detail the expression of BH in rat tissues, tissue extracts were subjected to SDS-PAGE followed by Western blotting, as shown in Fig. 2A. A protein band of BH with identical molecular mass was ubiquitously detected in the extracts from all rat tissues. Furthermore, Fig. 2B shows that the same protein band was detected in the extracts from circulating cells such as erythrocytes and leukocyte-rich cells in adult rat blood as well as in the extracts from rat hemo-lymphocytic cell lines. To estimate the amount of BH in rat tissues, a simple and sensitive ELISA was developed using a microtiter plate-based sandwich technique with a rabbit anti-rat BH IgG. The assay range was 20–600 ng/ml, with within-run and

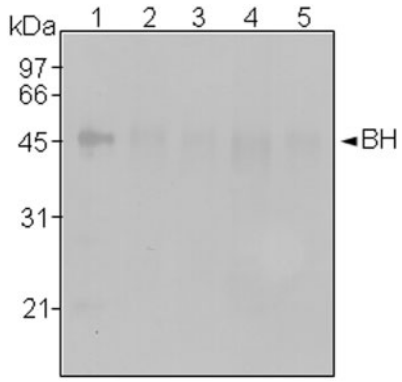

Fig. 1. **Specificity of rabbit anti-rat BH antiserum for papain family proteins.** Cathepsins B, H, and L were partially prepared from rat liver, and BH was purified from rat skin as described in 'Materials and Methods'. Papain was obtained from Sigma. The samples, in which an equal volume of BH-rich fraction prepared from rat liver was added to cathepsins B, H, and L, and papain solutions, were subjected to SDS-PAGE (12.5% gel) and then Western blotting was performed by the method described in 'Materials and Methods'. 1, BH; 2, cathepsin B; 3, cathepsin H; 4, cathepsin L; 5, papain. The migration position of BH is indicated. The numbers on the left side indicate the migration positions of marker proteins.

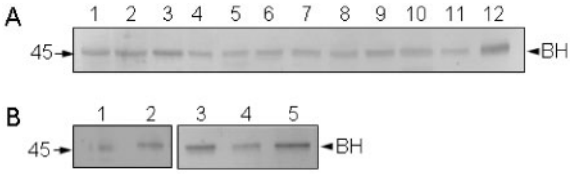

Fig. 2. **Western blotting of BH.** Extracts were prepared from rat tissues, circulating cells from blood and cell lines, and then SDS-PAGE and Western blotting were performed by the methods described under 'Materials and Methods'. (A) Supernatant extracts (5 μl) from rat tissues at 6 weeks old. Lane 1, kidney; 2, liver; 3, spleen; 4, lung; 5, stomach; 6, cerebrum; 7, uterus; 8, ovary; 9, testis; 10, skeletal muscle; 11, heart; 12, newborn skin. (B) Extracts (10 μl) from erythrocytes and leukocyte-rich cells of adult rat blood, and extracts (2 μl) from cell lines. Lane 1, leukocyte-rich cells; 2, erythrocytes; 3, Shay cells; 4, RBL-2H3 cells; 5, Y3-ag1.2.3 cells. The migration position of BH is indicated. The numbers of left side indicate the migration positions of ovalbumin.

Table 1. **The amount of BH in tissue extracts from male rat at 6 weeks at age.**

Male rat tissues ($n=6$)	Mean value ± SD (ng BH/mg tissue)
Brain[a]	13.7 ± 1.2
Heart	25.1 ± 1.3
Kidney	117.5 ± 27.5
Liver	106.7 ± 18.2
Lung	44.4 ± 6.6
Pancreas	3.7 ± 1.7
Skeletal muscle	64.7 ± 14.2
Spleen	74.7 ± 4.8
Stomach	34.6 ± 8.5
Testis	35.5 ± 11.6
Ovary[b]	14.3 ± 3.1
Uterus[b]	14.4 ± 4.0
Skin[c]	131.3 ± 14.6

[a]Cerebrum and cerebellum.
[b]Obtained from female rats aged 6 weeks old ($n=6$).
[c]Obtained from newborn rats ($n=7$).

between-run coefficients of variation of 7.0% ($n=10$) and 10.5% ($n=10$), respectively. The amount of BH in the rat tissues at 6 weeks old is summarized in Table 1. The newborn rat skin had the highest amount of BH. In male rats, the kidney and liver contained comparatively high levels of BH, while the amount in other tissues such as skeletal muscle, spleen, lung, stomach and testis was lower than that in the kidney. The pancreas had the lowest level of BH. In female rats, the amount of BH in the kidney was higher than that in other tissues. This pattern was similar to that in male rats. To examine the change in the amount of BH during aging, we estimated the amount in several tissues (brain, kidney, liver and spleen) from newborn male rats at 3 days after birth and from male rats at 6, 23 or 103 weeks old (Fig. 3). The amount of BH was increased during the 6 weeks after birth in the kidney, liver, and spleen, and the amount then tended to gradually decrease in them as the rats aged. However, the BH level in the brain showed no change during aging.

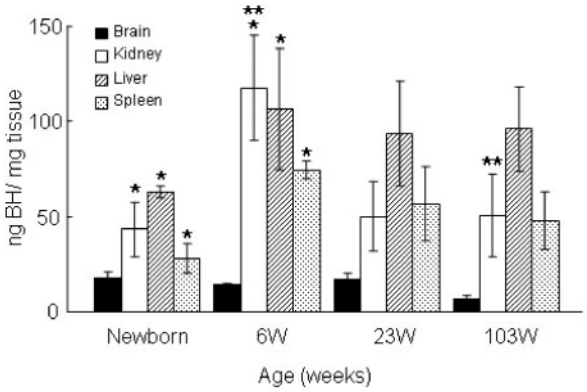

Fig. 3. **Alteration of the amount of BH in various tissues of male rats during aging.** The amount of BH in rat tissues at different ages (newborn after birth and 6, 23 and 103 weeks) were determined by ELISA. Each column and bar represent the mean ± SD of five or six rats. We examined differences in the mean levels of BH among ages using two-way analysis of Student's *t*-test. The asterisks indicate that the difference in the amount of BH is significant between the two values at newborn and 6 weeks in kidney, liver and spleen (*$P<0.01$), and between the two values at 6 weeks and 103 weeks in kidney (**$P<0.01$).

In other tissues from the oldest rats, the amount of BH was slightly decreased compared with that in the tissues of 6-week-old rats.

Here, BH was purified from the newborn rat epidermis, and it was shown that the stratus corneum cells and hair follicles of the skin as well as supraepithelial cells of the esophagus were strongly stained by an anti-rat BH IgG (*5*). It was, therefore, reasonable that we detected a high level of BH protein in the extract of newborn rat skin by western blotting and ELISA. We found by ELISA that BH was also present at relatively high levels in the extracts of rat kidney and liver. It was reported that BH plays an essential role in the metabolism of bleomycin *in vivo* and that the susceptibility of mammalian tissues to bleomycin-induced toxicity depends on the level of enzyme activity (*33–35*). The present results revealed that the tissues, such as liver and kidney, known to be resistant against the side-effects of bleomycin contained large amount of BH. On the other hand, the lung is the most susceptible tissue to bleomycin-induced toxicity (*36, 37*), but it contained a relatively large amount of BH protein, although the BH mRNA expression level in the lung was relatively low. This result suggests that resistance against bleomycin therapy in these tissues may not depend only on BH content, which is in accord with the fact that several risk factors of bleomycin-induced pneumonitis have been reported, including a high cumulative bleomycin dose, a decreased glomerular filtration rate and the chemotherapeutic method (*38*). The ELISA method was applied to determine the amount of BH in several rat tissues (brain, kidney, liver and spleen) during aging. The amount of BH in the kidney, liver and spleen, but not the brain, increased significantly during the 6 weeks after birth, and then tended to decrease as the rats aged. Recently, several investigators have reported that the half-life of cellular proteins is

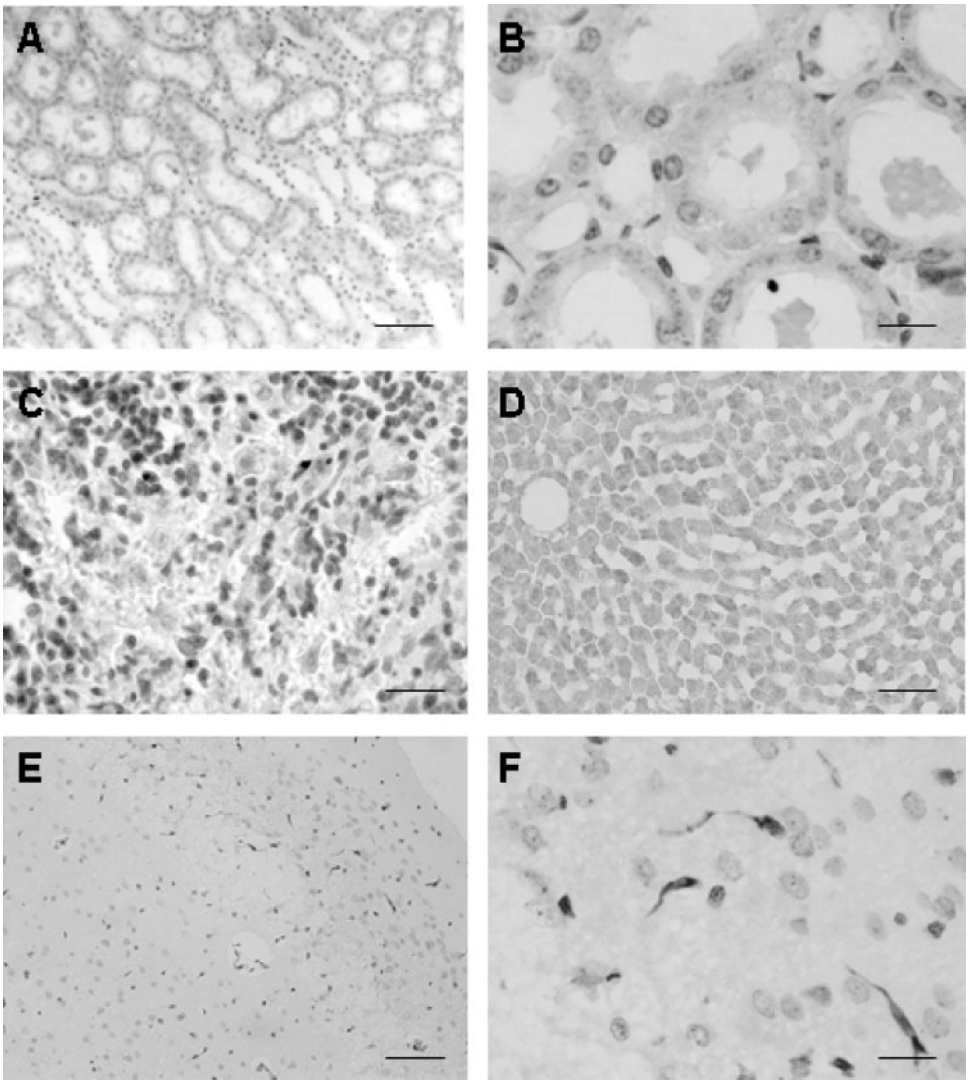

Fig. 4. **Immunohistochemical localization of BH in rat tissues.** (A) Kidney. The cortex of the kidney was stained positively. (B) High magnification of (A). Granular immunoreactivity was observed in the cytoplasm of epithelial cells of both distal and proximal tubular cells. (C) Spleen. BH-positive staining cells were present in the red pulp. (D) Liver. BH was positive in the cytosol of hepatocytes. (E) Brain. Neuronal and/or non- neuronal cells were positive after pretreatment with trypsin. (F) High magnification of (E). The scale bars indicate (A) 20 μm, (B) 5 μm, (C) 6.67 μm, (D) 6.67 μm, (E) 20 μm, and (F) 6.67 μm.

extended in senescent animals, suggesting that the proteolytic system is affected as animals age (*39–42*). In addition, both increases in synthesis and decreases in breakdown contribute to the accumulation of proteins important for the growth of the kidney, and abnormal proteolysis via the ubiquitin/proteasome ATP-requiring system is an important component of the pathogenesis of many kidney diseases (*43, 44*). The slight decrease of BH levels in the tissues of rats of advancing age may reflect a decrease of protein breakdown in protein metabolism, although the physiological role of BH is still unknown. Since there are several lines of evidence showing that BH is able to interact with essential protein factors related to protein metabolism in cells (*24, 45*), BH may play an ubiquitous physiological role in protein metabolism in rat tissues.

Immunocytochemistry of BH in Rat Tissues—We examined the localization of BH in the kidney, spleen, liver and brain, as these organs have the common feature that they seem to lack typical stratified epithelia, as shown by immunohistochemistry (Fig. 4). Significant immunostaining was detected at the cortex of the kidney, in which granular immunoreactivity was observed in the cytoplasm of epithelial cells of both the distal and proximal tubular cells (Fig. 4A and B). Immunoelectron microscopic examination showed immunoreactivity in the cytoplasm and also at some parts of the outer membrane of the rough endoplasmic reticulum of tubular epithelial cells of the kidney (Fig. 5A). In the liver, significant immunoreactivity was observed in the cytoplasm of hepatocytes near the central vein (Fig. 4D). Immunoelectron microscopic examination showed

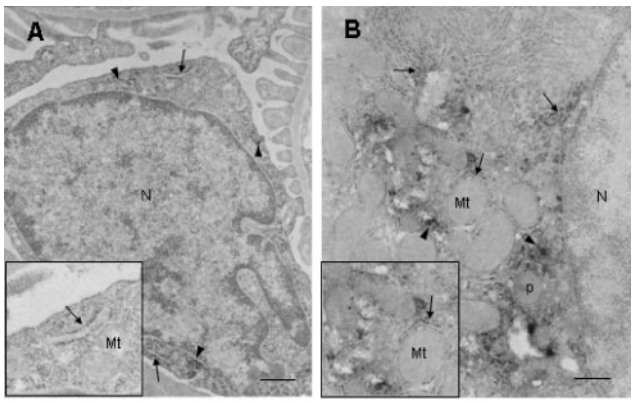

Fig. 5. **Immunoelectron micrographs of rat kidney and liver sections.** (A) Kidney. Immunoreactive particles were detected in the cytosol (arrowheads) and at the outer membrane of the rough endoplasmic reticulum (arrows). (B) Liver. BH-positive particles were mainly observed in the cytosol (arrowheads) and at the outer membrane of the rough endoplasmic reticulum (arrows). Higher-magnification images are magnified 2.3 and 1.5 times for kidney and liver, respectively. N, nuclei; Mt, mitochondria; p, peroxisomes. The scale bars indicate (A) 0.045 μm and (B) 0.05 μm.

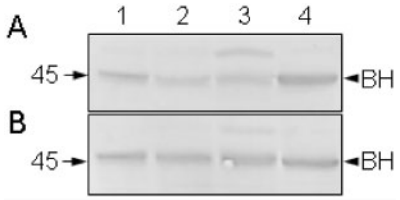

Fig. 6. **Western blotting of BH in subcellular fractions from rat liver and kidney.** Liver and kidney tissues were homogenized in 10 volumes of isotonic buffer by addition of sucrose (0.25 M final concentration) in a Teflon homogenizer. Subcellular fractionation was accomplished by sequential differential centrifugation to yield pellets of non-disrupted tissues and cellular nuclei (1000*g* for 10 min) and mitochondria (10,000*g* for 20 min). The mitochondrial supernatant was centrifuged at 100,000*g* for 60 min using a RP65T rotor in a Hitachi 65P-7 ultracentrifuge to yield a microsomal pellet containing ribosome and cytosolic supernatant fractions. The average overall enzyme activity recoveries of mitochondrial, microsomal and cytosolic supernatant fractions were 43.1, 69.4 and 72.4%, respectively, for liver and 44.1, 61.5 and 72.0%, respectively, for kidney. SDS-PAGE and Western blotting were performed using the above samples by the methods as described in 'Materials and Methods'. (A) Liver; (B) Kidney. Approximately 55 μg of total protein was applied per lane: lane 1, precipitate at 1000*g* for 10 min; lane 2, precipitate at 10,000*g* for 20 min; lane 3, precipitate at 100,000*g* for 60 min, lane 4, supernatant at 100,000*g* for 60 min. The migration position of BH is indicated. The numbers on the left side indicate the migration positions of ovalbumin.

immunoreactivity mainly in the cytoplasm as well as at the outer membrane of the rough endoplasmic reticulum of hepatocytes, in which the nuclei, mitochondria, and peroxisomes in the cytoplasm were devoid of BH immunoreactivity (Fig. 5B). These results may indicate that the subcellular localization of BH in the kidney and liver is essentially similar. Koldamova *et al*. demonstrated that human BH interacts with the homologues of rat ribosomal proteins L11 and L29 using a yeast two-hybrid system (*45*). Tissue extracts from the liver and kidney were prepared in isotonic buffer containing 0.25 M sucrose and separated into nuclear (1000*g* for 10 min), mitochondrial (10,000*g* for 20 min), microsomal (100,000*g* for 60 min) and cytosolic supernatant (100,000*g* for 60 min) fractions by differential centrifugation. The recoveries of marker enzyme activities of mitochondrial, microsomal and cytosolic supernatant fractions were 43.1, 69.4 and 72.4%, respectively, for liver and 44.1, 61.5 and 72.0%, respectively, for kidney. BH was found mostly in the cytosolic supernatant from liver and kidney (Fig. 6A and B). In addition, BH was significantly detected in the microsomal and mitochondrial fractions from both homogenates, but the detection in the latter fraction might have resulted from insufficient washing with isotonic buffer, judging from the low recovery. Furthermore, another weak band with slightly higher molecular mass than that of the BH subunit was usually detected in the microsomal fraction despite the lack of detection in the whole homogenate from rat tissues. Also, staining with second antibody alone yielded only negligible bands (data not shown). These results suggested that the localization of BH is mainly in the cytoplasm and is due to the interaction of BH with some component(s) such as ribosomal protein in the cells. In the spleen, BH immunostaining was detected in erythrocyte- and/or leukocyte-like cells in the red pulp, but no staining was observed in germinal cells in the white pulp (Fig. 4C). It would be of interest to know whether BH has a physiological role in the immune system, such as a role in hemo-lymphocytic lineage tissues and/or cells. Here, we detected BH protein in extracts of leukocyte-rich cells from rat blood and hemo-lymphocytic lineage cell lines by Western blotting (Fig. 2B). Furthermore, previous biochemical studies indicated that BH participates in the trimming of peptides that act as antigens in the major histocompatibility complex (MHC) class I processing pathway (*46, 47*). Recently, we reported that BH processed limited-length peptides such as β-amyloid peptides by endo-peptidase, amino-peptidase or caboxyl-peptidase activities (*22*). Also, we observed the BH immunoreactivity mainly in neurons and in dystrophic neuritis of senile plaques in Alzheimer's disease brains (*48*). Here, specific immunostaining was detected in neuronal and/or non-neuronal cells at the cortex of the cerebrum only when using sections pretreated with trypsin (Fig. 4E and F). These results suggest that BH protein in the brain may be masked with some cellular protein component(s) and may play a role in protein metabolism.

In summary, the results presented in this study suggest that the ELISA method developed here will be a useful tool for future studies on the physiological role of BH. Furthermore, the localization of BH immunoreactivity in the rat kidney, liver, spleen and brain suggest that BH is distributed in the cytoplasm and/or at the outer membrane of the rough endoplasmic reticulum together with some protein component(s). We speculate that BH participates in protein metabolism at a position downstream of the main protein

degradation systems such as the ubiquitin-proteasome and/or calpain systems, and in processing to functional proteins in mammalian cells.

This study was supported in part by a grant from Sagami Women's University. We are grateful to Prof. M. Isemura of the University of Shizuoka for providing the cultured cells. We also thank Dr J. Itoh of Tokai University for his excellent photographic assistance.

REFERENCES

1. Umezawa, H., Hori, S., Sawa, T. *et al.* (1974) Bleomycin-inactivating enzyme in mouse liver. *J. Antibiot. Ser.A* **27**, 419–424
2. Nishimura, C., Tanaka, N., Suzuki, H. *et al.* (1987) Purification of bleomycin hydrolase with a monoclonal antibody and its characterization. *Biochemistry* **26**, 1574–1578
3. Sebti, S.M., DeLeon, J.C., Lazo, J. (1987) Purification, characterization, and amino acid composition of rabbit pulmonary bleomycin hydrolase. *Biochemistry* **26**, 4213–4219
4. Rhyu, M.R., Nishimura, T., Kato, Y. *et al.* (1992) Purification and properties of aminopeptidase H from chicken skeletal muscle. *Eur. J. Biochem.* **208**, 53–59
5. Takeda, A., Higuchi, D., Yamamoto, T. *et al.* (1996) Purification and characterization of bleomycin hydrolase, which presents a new family of cysteine proteases, from rat skin. *J. Biochem.* **119**, 29–36
6. Sebti, S.M., Mignano, J.E., Jani, J.P. *et al.* (1989) Bleomycin hydrolase: Molecular cloning, sequencing, and biochemical studies reveal membership in the cysteine proteinase family. *Biochemistry* **28**, 6544–6548
7. Takeda, A., Masuda, Y., Yamamoto, T. *et al.* (1996) Cloning and analysis of cDNA encoding rat bleomycin hydrolase, a DNA-binding cysteine protease. *J. Biochem.* **120**, 353–359
8. Ferrando, A.A., Velasco, G., Campo, E. *et al.* (1996) Cloning and expression analysis of human bleomycin hydrolase, a cysteine proteinase involved in chemotherapy resistance. *Cancer Res.* **56**, 1746–1750
9. Brömme, D., Rossi, A.B., Smeekens, S.P. *et al.* (1996) Human bleomycin hydrolase: Molecular cloning, sequencing, functional expression, and enzymatic characterization. *Biochemistry* **35**, 6706–6714
10. Adachi, H., Tsujimoto, M., Fukasawa, M. *et al.* (1997) cDNA cloning and expression of chicken aminopeptidase H, possessing endopeptidase as well as aminopeptidase activity. *Eur. J. Biochem.* **245**, 283–288
11. Berti, P.J. and Storer, A.C. (1995) Alignment/phylogeny of the papain superfamily of cysteine proteases. *J. Mol. Biol.* **246**, 273–283
12. Ferrando, A.A., Pendas, A.M., Llano, E. *et al.* (1997) Gene characterization, promoter analysis, and chromosomal localization of human bleomycin hydrolase. *J. Biol. Chem.* **272**, 33298–33304
13. Kambouris, N.G., Burker, D.J., Creutz, C.E. (1992) Cloning and characterization of a cysteine proteinase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **267**, 21570–21576
14. Enenkel, C. and Wolf, D.H. (1993) *BLH1* codes for a yeast thiol aminopeptidase, the equivalent of mammalian bleomycin hydrolase. *J. Biol. Chem.* **268**, 7036–7043
15. Magdolen, U., Müller, G., Magdolen, V. *et al.* (1993) A yeast gene (*BLH1*) encodes a polypeptide with high homology to vertebrate bleomycin hydrolase, a family member of thiol proteinases. *Biochem. Biophys. Acta* **1171**, 299–303
16. Xu, H.E. and Johnston, S.A. (1994) Yeast bleomycin hydrolase is a DNA-binding cysteine protease. Identification, purification, biochemical characterization. *J. Biol. Chem.* **269**, 21177–21183
17. Yin, Z., He, J.Y., Gong, Z. *et al.* (1999) Identification of differentially expressed genes in Con A-activated carp (*Cyprinus carpio* L.) leukocytes. *Comp. Biochem Physiol.* **B124**, 41–50
18. Chapot-Chartier, M-P., Nardi, M., Chopin, M-C. *et al.* (1993) Cloning and sequencing of *pepC*, a cysteine aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris* AM2. *Appl. Environ. Microbiol.* **59**, 330–333
19. Fernández, L., Bhowmik, T., Steele, J.L. (1994) Characterization of the *Lactobacillus helveticus* CNRZ32 pepC gene. *Appl. Environ. Microbiol.* **60**, 333–336
20. Chapot-Chartier, M-P., Rul, F., Nardi, M. *et al.* (1994) Gene cloning and characterization of PepC, a cysteine aminopeptidase from *Streptococcus thermophilus*, with sequence similarity to the eukaryotic bleomycin hydrolase. *Eur. J. Biochem.* **224**, 497–506
21. Takeda, A., Nonaka, M., Ishikawa, A. *et al.* (1999) Immunohisto-chemical localization of the neutral cysteine protease bleomycin hydrolase in human skin. *Arch Dermatol. Res.* **291**, 238–240
22. Kajiya, A., Kaji, H., Isobe, T. *et al.* (2006) Processing of amyloid beta-peptides by neutral cysteine protease bleomycin hydrolase. *Pept. Prot. Lett.* **13**, 119–123
23. Lupas, A., Flanagan, J.M., Tamura, T. *et al.* (1977) Self-compartmentalizing proteases. *Trends Biochem. Sci.* **22**, 399–404
24. Koldamova, R.P., Lefterov, I.M., Disabella, M.T. *et al.* (1998) An evolutionarily conserved cysteine protease, human bleomycin hydrolase, binds to the human homologue of ubiquitin-conjugating enzyme 9. *Mol. Pharmacol.* **54**, 954–961
25. Towatari, T., Kawabata, Y., Katsunuma, N. (1977) Crystallization and properties of cathepsin B from rat liver. *Eur. J. Biochem.* **102**, 279–289
26. Schwartz, W.N. and Barrett, A.J. (1980) Human cathepsin H. *Biochem. J.* **191**, 487–497
27. Kirschke, H., Langner, J., Wiederanders, B. *et al.* (1977) Cathepsin L, a new proteinase from rat liver lysosomes. *Eur. J. Biochem.* **74**, 293–303
28. Lowry, O.H., Rosebrough, N.J., Farr, A.J. *et al.* (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265–271
29. Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
30. Schoner, W., von llberg, C., Kramer, R. *et al.* (1967) On the mechanism of Na^+- and K^+-stimulated hydrolysis of adenosine triphosphate. I. Purification and properties of a Na^+- and K^+- activated ATPase from ox brain. *Eur. J. Biochem.* **1**, 334–343
31. Baginski, E.S., Foá, P.P., Zak, B. (1974) Glucose-6-phosphatase. In *Methods of enzymatic analysis* (Bergmeyer, H. U., ed.) pp. 876–880, Verlag Chemie Weinheim, Weinheim, Germany
32. Megraw, R.E. (1971) Reaction of reduced nicotinamide adenine dinucleotide with 2,4-dinitropheneylhydradine in serum lactate dehydrogenase assays. *Am. J. Clin. Pathol.* **56**, 225–226
33. Filderman, A.E., Genovese, L.A., Lazo, J.S. (1988) Alternations in pulmonary enzymes following systemic bleomycin treatment in mice. *Biochem. Pharmaco.* **37**, 1111–1116
34. Sebti, S.M., Jani, J.P., Mistry, J.S. *et al.* (1991) Metabolic inactivation: A mechanism on human tumor resistance to bleomycin. *Cancer Res.* **51**, 227–232
35. Carter, S.K. (1985) *Bleomycin Chemotherapy* (Sikic, B.I., Rozencweig, M., and Carter, S.K., eds.) Ch.1, pp. 3–35, Academic Press, New York
36. Sikic, B.I. (1985) *Bleomycin Chemotherapy* (Sikic, B.I., Rozencweig, M., and Carter, S.K., eds.) Ch. 20, pp. 247–254, Academic Press, New York

37. Lazo, J.S. and Humphreys, C.J. (1983) Lack of metabolism as the biochemical basis of bleomycin-induced pulmonary toxicity. *Proc. Natl Acad. Sci. USA* **80**, 3054–3068
38. Jules-Elysee, E.K. and White, D.A. (1990) Bleomycin-induced pulmonary toxicity. *Clin. Chest. Med.* **11**, 1–20
39. Reznick, A.Z., Dovrat, A., Rosenfelder, L. *et al.* (1985) *Modification of Proteins During Ageing* (Adelman, R.C., Dekker, E.E., and Alan, R.L, eds.) pp. 69–81, New York
40. Richardson, A. and Cheung, H.T. (1982) The relationship between age-associated changes in gene expression, protein turnover, and the responsiveness of an organism to stimuli. *Life Sci.* **31**, 605–613
41. Ohtsuka, H., Takahashi, R., Goto, S. (1995) Age-related accumulation of high-molecular-weight ubiquitin protein conjugates in mouse brains. *J. Geronal.* **50A**, B277–B281
42. Hayashi, T. and Goto, S. (1998) Age-related changes in the 20S and 26S proteasomes activities in the liver of male F344 rat. *Mech. Ageing Dev.* **102**, 55–66
43. Franch, H.A. (2002) Pathways of proteolysis affecting renal cell growth. *Curr. Opin. Nephrol. Hypertens.* **11**, 445–450
44. Debigare, R. and Price, S.R. (2003) Proteolysis, the ubiquitin-proteasome system, and renal diseases. *Am. J. Renal Physiol.* **285**, F1–F8
45. Koldamova, R.P., Lefterov, I.M., DiSabella, M.T. *et al.* (1999) Human bleomycin hydrolase binds ribosomal proteins. *Biochemistry* **38**, 7111–7117
46. Stoltze, L., Schirle, M., Schwarz, G. *et al.* (2000) Two new proteases in the MHC class I processing pathway. *Nat. Immunol.* **1**, 413–418
47. Saveanu, L., Fruci, D., von Endert, P.M. (2002) Beyond the proteasome: trimming, degradation and generation of MHC class I ligands by auxiliary proteases. *Mol. Immunol.* **39**, 203–215
48. Nanba, Y., Ouchi, Y., Takeda, A. *et al.* (1999) Bleomycin hydrolase immunoreactivity in senile plaque in the brains of patients with Alzheimer's disease. *Brain Res.* **830**, 200–202