# Human Bleomycin Hydrolase: Molecular Cloning, Sequencing, Functional Expression, and Enzymatic Characterization

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ABSTRACT: We have cloned the cDNA of human bleomycin hydrolase (hBH), a protease which is thought to be involved in the metabolic inactivation of the antineoplastic drug bleomycin. The open reading frame consists of 1365 base pairs and is predicted to encode a 52 kDa protein. The protein shares 40% identity with yeast bleomycin hydrolase and contains the conserved active site residues (Cys, His, Asn) characteristic for cysteine proteases of the papain superfamily. Human bleomycin hydrolase has been functionally expressed in *Spodoptera frugiperda Sf*9 cells using the *Autographa californica* nuclear polyhedrosis virus. The 52 kDa recombinant protein forms a hexamer of 310 kDa and acts strictly as an aminopeptidase with a broad substrate specificity. The lack of a leader sequence and its pH optimum at 7.2 suggest a cytosolic/nuclear localization. Human bleomycin hydrolase was detected at low to moderate expression levels in most of the human organs tested. Significantly higher RNA levels have been observed in a variety of tumor cell lines. The human enzyme effectively degrades both forms of bleomycin (A2 and B2) *in vitro* and could indeed be responsible for the resistance of various tumors to this widely used anticancer drug.

Bleomycin is an antineoplastic glycopeptide antibiotic originally isolated from *Streptomyces verticillus* (Lazo & Sebti, 1989). Major indications for therapy with bleomycin are squamous cell carcinoma, lymphomas, and testicular and uterine carcinomas (Carter, 1985). In contrast to other anticancer drugs, bleomycin lacks typical side effects such as immunosuppression and myelosuppression (Umezawa, 1971), but drug resistance in various human tumors and pulmonary toxicity limit its broader application (Young, 1989). One possible mechanism of tumor resistance to bleomycin is the enzymatic inactivation of the drug. Several authors have demonstrated that a cysteine protease inhibitor-sensitive activity is involved in the inactivation of bleomycin. A major candidate is a thiol-dependent aminopeptidase, called bleomycin hydrolase, originally isolated from mice and rabbits (Umezawa et al., 1974; Sebti et al., 1987, 1989; Nishimura et al., 1987). This protease belongs to the papain superfamily (Sebti et al., 1989; Enenkel & Wolf, 1993) which is characterized by the conserved active site residues cysteine, histidine, and asparagine. To date, complete nucleotide/amino acid sequences of the aminopeptidase are only known from yeast (Enenkel & Wolf, 1993) and *Lactoccocus* (Chapot-Chartier et al., 1993). A partial sequence from the rabbit enzyme was published by Sebti et al. (1989). Bleomycin hydrolase is capable of inactivating bleomycin by a desamidation reaction in the $\beta$-aminoalanine moiety (Umezawa et al., 1974) and is inhibited by E-64, a general inhibitor of cysteine proteases (Sebti et al., 1989; Enenkel & Wolf, 1993). *In vivo* inhibition of bleomycin degradation by E-64 potentiated the antitumor activity of bleomycin (Sebti et al., 1991). In addition, overexpression of yeast bleomycin hydrolase induced resistance of *Saccharomyces cerevisiae* (Enenkel & Wolf, 1993) and NIH3T3 cells (Pei et al., 1995) to the antibiotic effect of bleomycin.

The relevance of these observations to the described resistance of various human tumors to bleomycin treatment remains speculative as long as the human equivalent of bleomycin hydrolase is unknown. In this paper we present for the first time the molecular cloning, sequencing, tissue distribution, heterologous expression, and enzymatic characterization of human bleomycin hydrolase, the human homologue of yeast bleomcin hydrolase, and compare the results with those obtained with the recombinant yeast enzyme. We also demonstrate the ability of human bleomycin hydrolase to degrade bleomycin and discuss the suitability of this protease as a potential drug target to overcome the bleomycin resistance of tumors.

## EXPERIMENTAL PROCEDURES

*Human Bleomycin Hydrolase cDNA.* The complete cDNA of human bleomycin hydrolase (hBH)[1] was cloned by PCR. An initial 486 bp PCR fragment was obtained from a human fetal lung Quick Clone cDNA pool (Clontech, Palo Alto,-CA) using degenerate primers derived from the partial rabbit cDNA sequence (Sebti et al., 1989): 5′ TGY TGG ATH TTY TCN TG 3′ and 5′ TA YTC CCA NGT RAA NGT YTC 3′. The 5′ end of the full length clone was obtained using the human lung 5′-RACE-Ready cDNA with an anchor primer (Clontech, Palo Alto, CA) and hAPH specific primers derived from the initial PCR product: 5′ GCA TAT CCC ATT GGC CAC CAT CAT TTG GAG GG 3′ and 5′GG CTC CTT TCT CTG GGC TGT GTC 3′. The latter primer,

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[1] Abbreviations: amu, atomic mass unit; hBH, human bleomycin hydrolase; MCA, methyl coumarylamide; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Z, (benzyloxy)carbonyl; yBH, yeast bleomycin hydrolase.

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located upstream from the first primer, was used to amplify the PCR signal in a secondary PCR reaction, giving the sequence of the initiation codon including 120 bp from the nontranslating 5′ end. The 3′ end was generated by PCR from the λgt11 human thymus 5′-stretch cDNA (Clontech, Palo Alto, CA) using the following primers: 5′ GAC ACC AGA CCA ACT GGT AAT G 3′ and 5′GGT AGC GAC CGG CGC TCA G 3′ as anchor primers located in the λgt11 vector and 5′ CCC AGG AGG GCA AGC CAA TCA CC 3′ and 5′ CCA GAA GAG CTC AGG GCG ATG C 3′ in the coding sequence of hBH. The latter primers of both pairs were used to amplify the PCR signal. An additional hBH specific primer (5′ GAC CCA CGC CAT GAC CTT CAC TG 3′) was used to amplify the complete 3′ coding end including a 140 bp stretch of the nontranslated region. PCR products were subcloned into the pCR-Script SK(+)vector (Stratagene, La Jolla, CA) and sequenced directly on a 373A DNA sequencer (Applied Biosystems, Foster City, CA). Full length clones of human bleomycin hydrolase cDNA were obtained by PCR reaction using Quick Clone cDNA from human fetal lung, fetal brain, and leukocytes and the following oligonucleotide primers: ′5 GCC T**AG ATC T**GG CGC CAT GAG CAG CTC GGG 3′ and 5′ AG**T CTA GA**T CAC TCA GCC AAA GCT CCC ATG G 3′. The primers contain a *Bgl*II and a *Xba*I site (in boldface) suitable for cloning into the pCL1396 *Baculovirus* expression vector. All PCR reactions were carried out using the proof reading *Pfu* polymerase (Stratagene, La Jolla, CA).

*Construction of Transfer Vector and Expression.* The 1.5-kb fragment containing a 5′*Bgl*II and a 3′*Xba*I site was inserted into the *Bgl*II and *Xba*I site of the pVL1392 transfer vector (PharMingen, San Diego, CA). Recombinant baculovirus was generated by homologous recombination following cotransfection of the baculovirus transfer vector and linearized *Ac*NPV genomic DNA into *Sf*9 cells (PharMingen, San Diego, CA). Pure virus (*Ac*NPVhBH) was obtained by plaque purification. Sf9 cells were grown in Sf900II media (Gibco BRL, Grand Island, NY) to a density of $2 \times 10^6$ cells/mL and infected at a moi of 1. Total cell number and enzymatic activity of the recombinant human bleomycin hydrolase were monitored every 24 h. After 3.5 days the cells were harvested.

*Cloning, Construction of the Transfer Vector, and Expression of Yeast Bleomycin Hydrolase.* Yeast bleomycin hydrolase (YBH) was cloned by PCR using the forward primer 5′-ATA**GGATCC**GTTAC**ATG**CTTCCTACTTCTG-3′ and the reverse primer 5′-CG**TCTAGATTA**TTTGGC-CAAAGCACCCA-3′, which contain the initiator methionine and termination codons and the *Bam*HI and *Xba*I restriction sites, respectively, as indicated in bold. PCR was carried out using Ultima DNA polymerase (Perkin-Elmer). The 1470 bp product was cloned into the *Bam*HI and *Xba*I restriction sites of pBluescript (Stratagene) and completely sequenced in both directions. Recombinant baculovirus was generated as described above following cloning of YBH into the *Bgl*II and *Xba*I site of the pVL1392 transfer vector (PharMingen, San Diego, CA).

*Northern Blot Hybridization.* Multiple tissue and cell line Northern blots (Clontech, Palo Alto, CA, calibrated against actin) were hybridized for 20 h at 42 °C in a hybridization buffer (5× SSPE, 10× Denhardt's solution, 50% formamide, 2% SDS, and 10 μg/mL denatured salmon sperm DNA) containing the [α-$^{32}$P] dCTP labeled 486 bp hBH fragment. The blots were washed in 2× SSC/0.1% SDS for 20 min at room temperature and twice at 68 °C for 20 min each.

*Purification.* The human and yeast homologues of bleomycin hydrolase were expressed and purified under the same conditions. The *Sf9* cells were harvested from the production media by centrifugation at 2000*g*, were brought up to 60 mL with 100 mM Tris-HCl, pH 7.6, containing 1 mM dithiothreitol and 1 mM $Na_2$EDTA and were lysed in a Dounce homogenizer. The cell lysate containing active human bleomycin hydrolase was cleared by centrifugation (16000*g*), and the supernatant was adjusted to pH 4.0 by addition of 1 M acetic acid. The precipitate was removed by centrifugation (16000*g*) and the supernatant was adjusted to 2.5 M ammonium sulfate in 50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and again cleared by centrifugation. The activities remaining in the supernatant were then loaded onto a butyl-Sepharose 4 Fast Flow column (Pharmacia, Uppsala, Sweden) which was washed with an ammonium sulfate gradient (2.5 to 0 M) in 50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 1 mM EDTA. The activity was eluted at 0.5 to 0 M ammonium sufate. The pooled and concentrated fractions were subsequently applied to an FPLC Mono Q column (Pharmacia, Uppsala, Sweden) and eluted with a linear NaCl gradient (0−0.5 M) in 20 mM Tris-HCl, pH 7.5, containing 0.5 mM dithiothreitol. The activities emerged from the column at a salt concentration between 0.25 and 0.32 M NaCl. Approximately 90% electrophoretically pure enzyme was obtained in a final sizing step using a Superdex 200 FPLC column (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.5 mM dithiothreitol.

*Molecular Mass Determination*. The molecular mass of the monomer was determined by SDS−PAGE in 4−20% Tris/glycine gels (Novex, San Diego, CA) using the Multimark protein mixture (Novex) as molecular weight standard. The mass of the oligomer was determined by gel filtration on a Superdex 200 FPLC column (Pharmacia, Uppsala, Sweden) using the following standard proteins (Pharmacia): thyroglobulin (669 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), and blue dextran for the void volume.

*Assays with Methyl Coumarylamide Substrates and Inhibitors.* Amino acid methyl coumarylamides (MCA) were purchased from Bachem Bioscience Inc. (King of Prussia, PA). L-3-Carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) and iodoacetamide were obtained from Sigma (St. Louis, MO).

Initial rates of substrate hydrolysis (substrate concentration 25 μM) were monitored in 1 cm cuvettes at 25 °C in a Perkin-Elmer fluorimeter at excitation and emission wavelengths of 380 and 450 nm, respectively. Recombinant human and yeast bleomycin hydrolases were assayed at a constant enzyme concentration (ca. 20 nM) in 100 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 1 mM $Na_2$-EDTA.

Inhibition of both aminopeptidases was assayed at a constant substrate (10 μM Cit-MCA) and enzyme concentration (approx. 20 nM, based on BSA protein calibration standard curve) in the presence of different inhibitor concentrations in the substrate assay buffer. Human and yeast bleomycin hydrolase, respectively, were preincubated with irreversibly acting inhibitors for 5 min, or the reaction was

started immediately for reversible inhibitors with substrate. Residual activity was monitored, and percent inhibition was calculated from the uninhibited rate.

The inactivation rates ($k_{obs}$) in the presence of substrate and for different inhibitor concentrations were determined for the aminopeptidases according to Tian and Tsou (1981). Progress curves for the inactivation of the proteases were monitored at room temperature (22 °C). By fitting the rate constants obtained at different inhibitor concentrations to eq 1, the inactivation parameters $K_{i,app}$ and $k_{inact}$ were obtained. The $K_{i,app}$ values were then corrected for competitive inhibition using eq 2.

$$k_{obs} = k_{inact}[I]/K_{i,app} + [I] \quad (1)$$

$$K_i = K_{i,app}/(1 + [S]/K_m) \quad (2)$$

*pH Activity Profile.* Initial rates of substrate hydrolysis were monitored as described above. The pH activity profiles of both aminopeptidases were obtained at 10 $\mu$M Arg-MCA ([S] < $K_m$ = 160 $\mu$M, where the initial rate $v_0$ is directly proportional to the $k_{cat}/K_m$ value). The following buffers were used for the pH activity profile: 100 mM sodium citrate (pH 2.8−5.6) and 100 mM sodium phosphate (pH 5.8−8.0). All buffers contained 1 mM EDTA and 0.4 M NaCl to minimize the variation in ionic strength. A three protonation model (Khouri et al., 1991) was used for least-squares regression analysis of the pH activity data. The data were fitted to the equation:

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)/([H^+]/K_1 + 1 + K_2/[H^+]) \quad (3)$$

*Tryptic Mapping and Sequence Determination.* Bleomycin hydrolase (340 pmol in 20 $\mu$L) was dissolved in 100 $\mu$L of 6 M guanidine hydrochloride, 0.1 M Tris, 1 mM $Na_2EDTA$, and 2 mM dithiothreitol (all from Sigma Chemical Co., St. Louis, MO) at pH 8.4, heated in a capped glass microvial (Hewlett-Packard, Palo Alto, CA) at 100 °C for 5 min, and carboxymethylated by 5 mM iodoacetate (Aldrich Chemical Co., Milwaukee, WI) at 30 °C for 30 min. The reaction was stopped by the addition of dithiothreitol to 5 mM, and the contents were flow-dialyzed against 0.05 M pH 8.0 ammonium bicarbonate (Sigma Chemical Co., St. Louis, MO). The protein was then digested for 24 h by 1:50 w/w trypsin (Worthington Biochemical Corp., Freehold, NJ), in the presence of 10 mM $CaCl_2$ to prevent autolysis of the trypsin. Peptides were isolated by reversed phase chromatography on a Hewlett-Packard 1090m liquid chromatograph using a 2.1 × 250 mm Vydac (The Separations Group, Hesperia, CA) C18 column, and 0.3 mL/min elution at 40 °C, using a gradient from 0−40% acetonitrile−0.1% v/v trifluoroacetic acid (Aldrich Chemical Co., Milwaukee WI) in 50 min. The three largest peaks were sequenced by the Edman degradation at the Protein Structure Lab (University of California, Davis, CA) by Dr. Jack Presley.

*Degradation of Bleomycin by Human Bleomycin Hydrolase and Human Cathepsins S and O2.* The hydrolysis of the sulfate salt of bleomycins A2 and B2 (obtained as a mixture from Sigma Chemical Co., St. Louis, MO) by bleomycin hydrolase and cathepsins S and O2 was examined by reversed phase chromatography. Purified bleomycin hydrolase (87 ng) was mixed with 34.5 nmol of bleomycin in 233 $\mu$L of 0.1 M Tris-HCl, pH 7.5, containing 2 mM dithiothreitol and 1 mM $Na_2EDTA$ at room temperature (23 °C). To examine the kinetics of hydrolysis, samples were injected about every 83 min, starting 2 min after mixing the reactants. Isocratic elution on a 2.1 × 250 mm Vydac (The Separations Group, Hesperia, CA) C18 column was at 7% acetonitrile−0.1% trifluoroacetic acid (buffer B) for 5 min, and then with an increasing gradient to 20% acetonitrile−0.1% trifluoroacetic acid over 50 min. An alternative elution used for the kinetics was isocratic elution at 7% buffer B for 50 min. The reaction of cathepsin S (0.53 or 3.2 $\mu$M) with 34.5 nmol of bleomycin was followed as above for 11 h at pH 6.5 and 7.5. The reaction of cathepsin O2 (160 nM) with bleomycin was followed at pH 7.5 for 13 h, and the reaction at pH 5.5 for 15 h was followed at cathepsin O2 concentrations of 16 and 320 nM, respectively. Recombinant human cathepsin S and O2 were expressed and purified as described in Brömme and McGrath (1996) and Brömme et al. (1996).

*Purification and Characterization of Bleomycins A2, B2, and Their Products.* Bleomycins A2 and B2 were separated from 2.1 mg of bleomycin (Sigma, St. Louis, MO) by isocratic elution at 7% buffer B on a 4.6 × 250 mm Vydac C18 reversed phase column eluted at 1%/min. A similar mixture was purified after reaction with bleomycin hydrolase for 30 h under conditions defined above; several time points were sampled to assure completion of the reaction. Samples of bleomycin A2 and its degradation product were dissolved in $D_2O$ (99.9 atom %, Aldrich, Milwaukee, WI) and were examined by proton NMR on a Jeol Eclipse 270, and by electrospray mass spectrometry at the College of Chemistry, University of California, Berkeley, CA.

## RESULTS

*Human Bleomycin Hydrolase cDNA and Amino Acid Sequence.* A 490 bp PCR fragment was obtained from a human fetal lung cDNA pool using degenerate primers derived from a partial rabbit sequence of bleomycin hydrolase (Sebti et al., 1989). The 5′ end of the complete human bleomycin hydrolase sequence including 120 bp from the nontranslating 5′ end was obtained using the 5′RACE cloning sytem and specific primers derived from the 490 bp fragment. The 3′ end was cloned by PCR using two different sets of internal hBH specific oligonucleotides and anchor primers derived from a $\lambda$gt11 human thymus 5′stretch cDNA. With the help of the first primer pair located in the initial 490 bp fragment, a variety of incomplete PCR fragmets were obtained. Most of the fragments stopped at base pair position 1014, identical to the 3′ end obtained from the rabbit fragment by Sebti et al. (1989), whereas a few clones gave extended sequences still missing 30 nucleotides to the putative stop codon. This may indicate that steric problems in the RNA sequence of human and rabbit bleomycin hydrolase result in the ligation of incomplete fragments into the appropriate cDNA libraries. The complete 3′ end including 140 bp of the nontranslating region was obtained with a second specific primer set derived from the sequence obtained from the first PCR 3′ product.

Full length clones of the hBH were generated by PCR from cDNA pools from human fetal lung, fetal brain, and peripheral blood leukocytes using primers designed from the nontranslating 3′ and 5′ ends. The cDNA of hBH encodes a 455 amino acid protein (Figure 1). The open reading frame

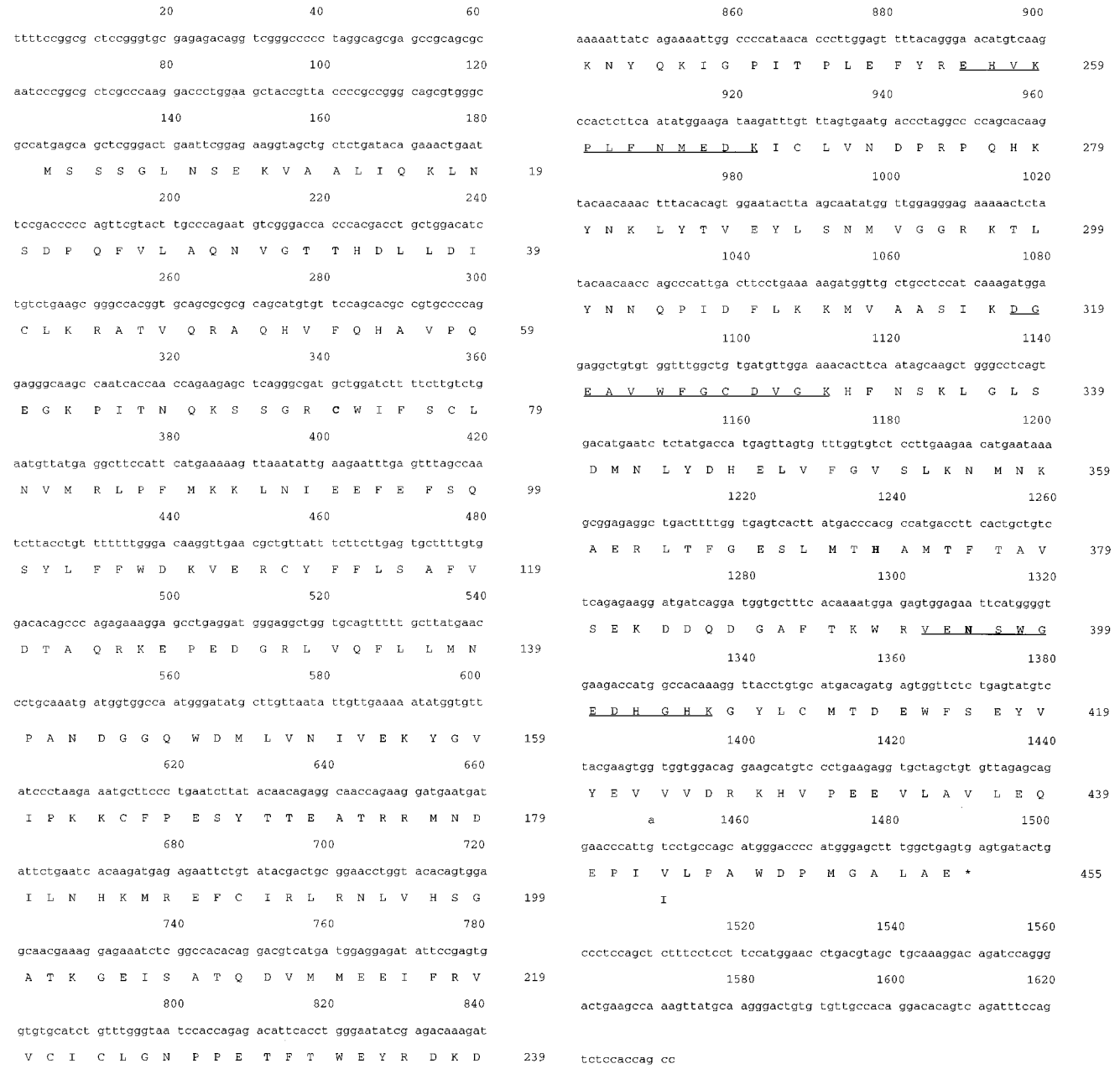


FIGURE 1: Nucleotide and protein sequence of the human bleomycin hydrolase. The active site residues cysteine, histidine, and asparagine are indicated in boldface. Sequences confirmed by N-terminal sequencing are underlined.

starts after a typical translation initiation sequence (Kozak, 1986) at nucleotide 123 with an ATG codon and ends at nucleotide 1491 with a TGA stop codon. No polyadenylation site was detected within the first 140 nucleotides 3′ of the stop codon. The deduced amino acid sequence contains no potential glycosylation sites.

Sequences obtained from three different human organs were identical with the exception of amino acid residue 443. Either a valine or an isoleucine was found in this position attributed to an A/G mutation in nucleotide position 1450 (Figure 1). Four out of six sequences obtained from the leukocyte cDNA (generated from a male/female pool) as well as from fetal lung cDNA (male/female pool) contained a valine residue in position 443. In addition, all six sequences derived from fetal human brain (single female) contained also a valine residue. Since both isoforms seem to be present in human individuals, a pathological consequence of this conservative mutation seems to be unlikely.

The absence of a signal sequence suggests that human bleomycin hydrolase has a cytosolic/nuclear localization.

The amino acid sequence of human bleomycin hydrolase exhibits highly conserved residues typical for the active site regions of cysteine proteases of the papain superfamily (Berti & Storer, 1995). These residues are the active site cysteine, histidine, asparagine, as well as a glutamine residue forming the putative oxyanion hole (residues in boldface in Figure 1). In contrast to papain-like cathepsins (135−165 amino acid residues), bleomycin hydrolase has a substantially longer stretch of amino acids (297 residues) between its active site cysteine and histidine residue. According to a recently solved X-ray structure (Joshua-Tor et al., 1995), most of these residues form two pairs of antiparallel α-helices extending from the catalytic domain which seem to be involved in the oligomerization of the protease.

*Tissue Distribution of Human Bleomycin Hydrolase.* Expression of human bleomycin hydrolase, detected by

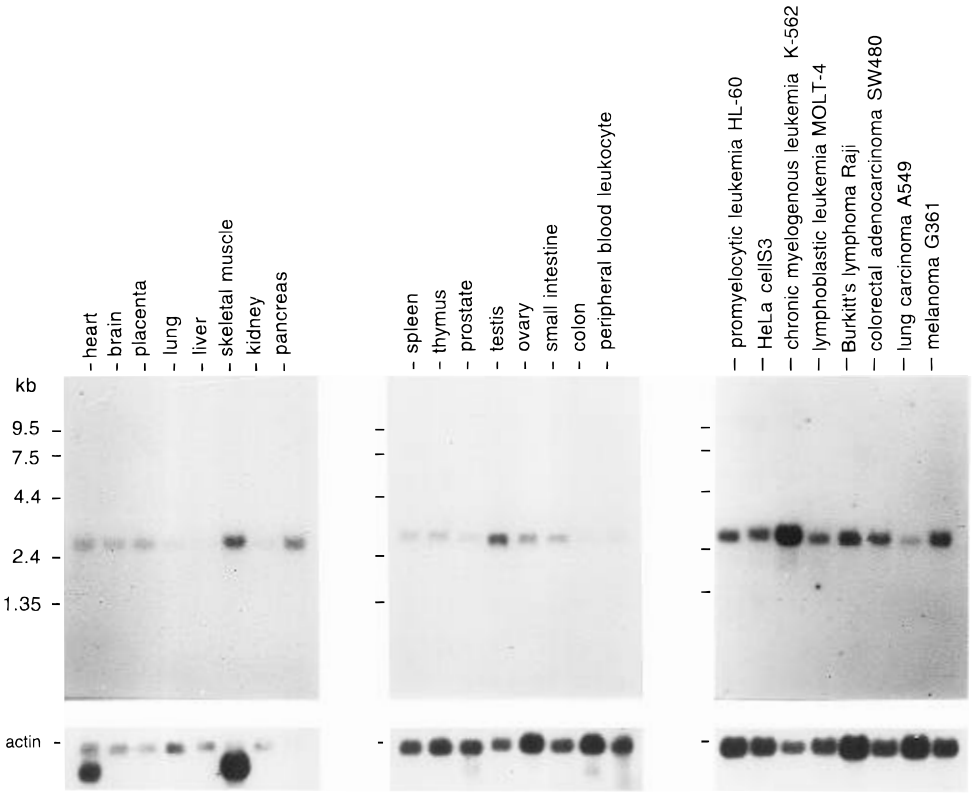


FIGURE 2: Northern blot analysis of human bleomycin hydrolase in human tissues and tumor cell lines. Nitrocellulose blots were hybridized with $^{32}$P-labeled probe of human bleomycin hydrolase. The lower panel shows the actin probing of the blots as a control.

Northern blot analysis, was at low to moderate levels in most organs tested (spleen, thymus, prostate, ovary, small intestine, heart, brain, placenta, lung). Elevated expression levels were observed in testis, skeletal muscle, and pancreas, and very low expression levels were seen in liver, kidney, colon, and peripheral blood leukocytes (Figure 2). Northern analysis of a variety of tumor cell lines revealed a high level of hBH expression in chronic myelogenous leukemia K-562 cells, moderate levels in promyelocytic leukemia HL-60, Hela S3, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, and melanoma G361 cells, and a low expression level in lung carcinoma A549 cells. The approximate size of bleomycin hydrolase mRNA on Northern blots is 2.5 kb, which is in accordance to the size described for the rabbit mRNA (Sebti et al., 1989).

*Expression and Enzyme Purification.* Human bleomycin hydrolase has been expressed in *Sf9* cells using the *Baculovirus* expression system. The expression level of the aminopeptidase was monitored as an Arg-MCA hydrolyzing activity inhibitable by 0.5 mM iodoacetic acid. The cells were harvested 3 days post-infection. A 55-fold purification with a yield of 45% was achieved after 3 chromatography steps (butyl-Sepharose 4; MonoQ; Superdex 200) resulting in a protein of approximately 90% purity (Table 1, Figure 3). Recombinant human bleomycin hydrolase displays a molecular mass of 310 kDa by gel filtration and 52 kDa by SDS−polyacryamide gel electrophoresis (Figure 4). This indicates that the active enzyme forms a hexamer as recently described for the yeast enzyme analog (Joshua-Tor et al., 1995). The N-terminus of the 52 kDa subunit is blocked, but 3 internal sequences obtained from a tryptic digest confirmed the protein sequence deduced from the cDNA (sequences 256−267, 318−330, 394−405; underlined in Figure 1).

Table 1: Purification of Human Bleomycin Hydrolase from the *Baculovirus* Expression System[a]

| assay | total protein (mg) | total act. (μmol/min) | sp act. [μmol/(mg·min)] | purifn factor | yield (%) |
|---|---|---|---|---|---|
| crude extract | 694 | 10332 | 14.9 | 1 | 100 |
| 2.4 M ammonium sulfate fractionation (SN) | 91 | 7740 | 85 | 5.7 | 75 |
| butyl-Sepharose 4 | 27 | 5998 | 222 | 15 | 58 |
| MonoQ | 20 | 5921 | 296 | 20 | 57 |
| S200 | 5.7 | 4644 | 815 | 55 | 45 |

[a] For details see Experimental Procedures.

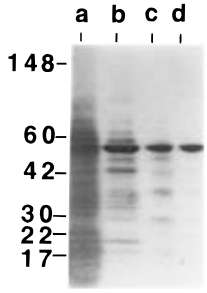


FIGURE 3: SDS−PAGE of purified recombinant human bleomycin hydrolase (Coomassie staining). Lane a, crude SF9 fraction; lane b, after passage through *n*-butyl Fast Flow; lane c, after passage through MonoQ; lane d, after passage through Superdex 200. Molecular mass standards are indicated in the left lane.

Yeast bleomycin hydrolase was expressed and purified under analogous conditions. Yields of expression and purification were comparable to those of the human protease.

*Enzymatic Characterization of Human Bleomycin Hydrolase.* Human bleomycin hydrolase has a pH optimum of pH 7.2 and is characterized by a narrow bell-shaped pH−activity profile (Figure 5). The neutral pH optimum of the

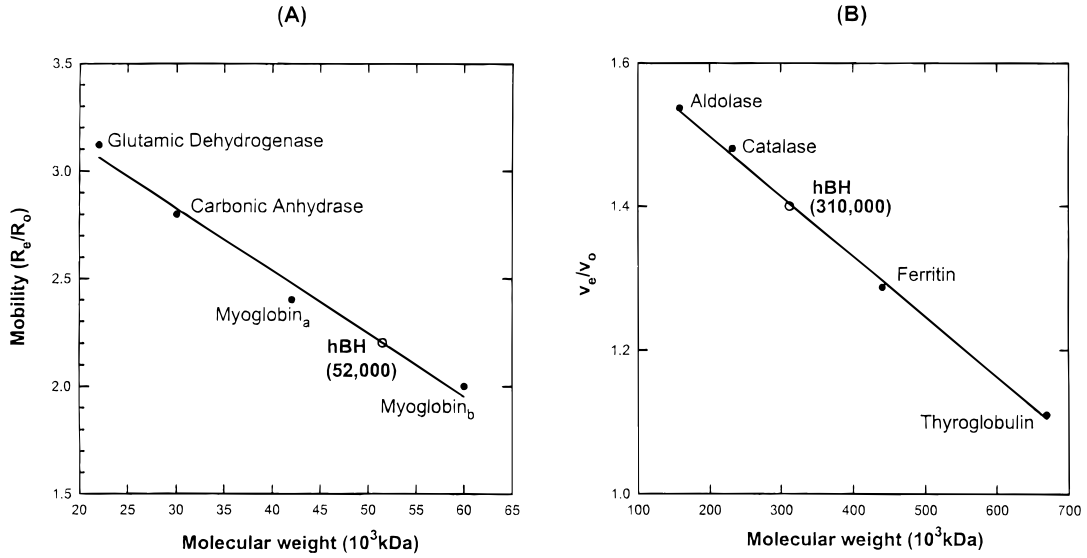


FIGURE 4: Measurement of molecular weight of human bleomycin hydrolase on (A) 4−20%Tris/glycine SDS−PAGE and (B) Superdex 200. (A) The mobilities of dye and proteins were indicated as $R_0$ and $R_e$, respectively. (B) The void volume is indicated as $V_0$, and the elution volumes are $V_e$.

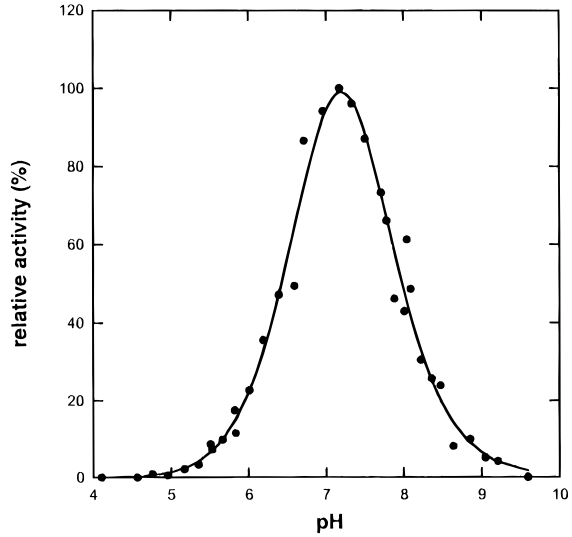


FIGURE 5: pH activity profile for recombinant human bleomycin hydrolase. The relative rate of hydrolysis was calculated from $k_{cat}/K_m$ values obtained by measuring the initial rates of Arg-MCA hydrolysis and by dividing by enzyme and substrate concentration.

Table 2: p*K* Values of pH Activity Profiles of Recombinant Human (hBH) and Yeast Bleomycin Hydrolase (yBH)

| protease | p$K_1$ | p$K_1$ | p$K_2$ | pH optimum |
|---|---|---|---|---|
| hBH | $5.24 \pm 0.25$ | $6.79 \pm 0.03$ | $7.55 \pm 0.01$ | 7.2 |
| yBH | $4.1 \pm 0.9$ | $6.89 \pm 0.03$ | $7.74 \pm 0.02$ | 7.3 |

protease is in accordance with its assumed cytosolic/nuclear localization which is derived from the absence of a signal peptide sequence in its gene structure. The pH activity profile of bleomycin hydrolase is very different from that of cysteine proteases such as papain or cathepsins S and O2. Whereas the observed p$K_2$ value of 7.55 (7.74 for the yeast analog; Table 2) is in the range of p$K_2$ values described for cathepsins (Brömme et al., 1993, 1996) and may reflect to some extent the active site histidine residue, the p$K_1$ values for the human and yeast enzyme (6.79 and 6.89) are very distinct from appropriate values determined for cathepsins (about 4−4.5). It remains unclear which amino residue(s) is/are responsible for the p*K* value observed in the neutral pH range. Using a three protonation model which results in a better fitting of the experimental data points**,** a third p*K* value can be described. This p*K*, between 4 and 5, resembles the p*K* corresponding to the active site cysteine residue in papain-like cysteine proteases. The low activity of the enzyme at acidic pH does not allow a greater accuracy in the determination of this p*K* value. However, it is also possible that the observed p*K* values represent functionalities of the substrate as well as of a substrate binding group within the enzyme. The p$K_2$ value could be assigned to an α-amino group of the aminopeptidase substrate whereas the p$K_1$ could represent a negatively charged group interacting with the α-amino group of the substrate. Interestingly, the crystal structure of the yeast bleomycin hydrolase reveals an interaction between the carboxylate group of the C-terminus of the protease with the α-amino group of the substrate. However, any assignments of p*K* values to particular residues within the protease or substrate remain speculative. Site-directed mutagenesis experiments may help to clarify these questions.

The substrate specificity of human bleomycin hydrolase was determined with a broad range of methyl coumarylamides and compared with the relative activities of yeast bleomycin hydrolase. Both the human and the yeast proteases exhibit no activity with N-protected peptide substrates such as Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, or Z-Val-Val-Arg-MCA, revealing that both enzymes do not have a substantial endopeptidase activity. In contrast, amino acid methyl coumarylamides are very effective substrates for both enzymes. Citrulline-MCA is the most efficiently hydrolyzed substrate by both enzymes. On the other hand, both species variants share very low rates of hydrolysis for β-branched amino acids such as valine and proline and for D-amino acid substrate derivatives (Figure 6). Furthermore, both enzymes are characterized by their inability to hydrolyze Asp-MCA but by the acceptance of Glu-MCA as a substrate. The cleavage of a glutamyl amino acid substrate was already demonstrated by Enenkel and Wolf (1992) for the yeast protease. Despite the observed similarities, both enzymes display some differences in their specificity. Human bleomycin hydrolase hydrolyzes methionine and leucine substrate

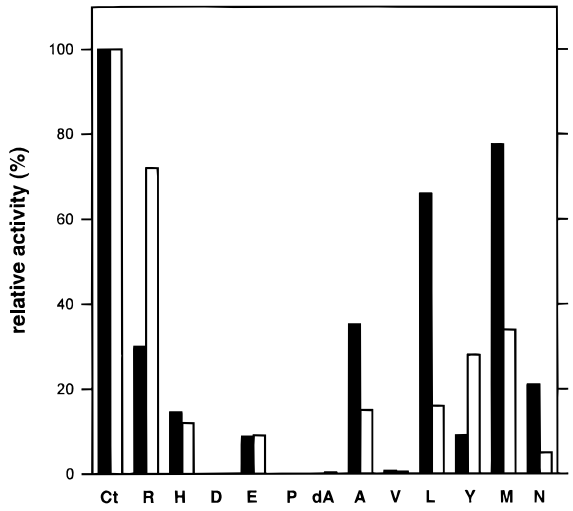


FIGURE 6: Relative activities for the hydrolysis of $NH_2$-AA-MCA substrates by human (black columns) and yeast bleomycin hydrolase (white columns). The following abbreviations are used: Ct, citrulline; R, arginine; H, histidine; D, aspartate; E, glutamate; P, proline; dA, D-alanine; A, alanine; V, valine; L, leucine; Y, tyrosine; M, methionine; N, asparagine.

Table 3: Inhibitor Profile of Human and Yeast Bleomycin Hydrolase

| | | | % inhibition | |
|---|---|---|---|---|
| | inhibitor | [inhibitor] | hBH | YBH |
| serine protease inhibitors | PMSF[a] | 1 mM | 0 | 0 |
| | befablock[a] | 0.2 mM | 0 | 0 |
| serine/cysteine protease inhibitors | leupeptin | 100 $\mu$M | 0 | 0 |
| | chymostatin | 10 $\mu$M | 0 | 0 |
| aspartate protease inhibitor | pepstatin | 0.1 $\mu$M | 0 | 0 |
| metalloprotease inhibitors | EDTA | 10 mM | 0 | 0 |
| | bestatin | 0.2 mM | 0 | 0 |
| cysteine protease inhibitors | iodoacetate[a] | 1 mM | 100 | 100 |
| | | | 20 $M^{-1}$ $s^{-1}$ [b] | 520 $M^{-1}$ $s^{-1}$ [b] |
| | E-64[a] | 50 $\mu$M | 47 | 100 |
| | | | 60 $M^{-1}$ $s^{-1}$ [b] | 1100 $M^{-1}$ $s^{-1}$ [b] |
| | NEM[a] | 500 $\mu$M | 84 | 100 |

[a] Preincubation of enzyme and inhibitor for 5 min. [b] Second-order rate constants of inactivation.

derivatives more efficiently than the yeast enzyme, whereas the latter protease shows a higher preference toward arginine and tyrosine substrates (Figure 6).

*Inhibitor Profile*. Both the human and the yeast recombinant bleomycin hydrolases are characterized by an inhibitor profile typical for cysteine proteases. Significant inhibition was obtained with cysteine protease inhibitors such as iodoacetic acid, E-64, and NEM (Table 3). Similar to differences observed in their substrate specificity, both enzymes exhibit differences in their rates of inactivation by inhibitors. In general, the yeast enzyme is more efficiently inhibited by most of the tested inhibitors than the human enzyme. N-Terminal protected peptide based cysteine protease inhibitors of the aldehyde as well as diazomethane class did not exhibit any inhibitory potency (data not shown). No inhibition was observed with general serine, aspartate, and metalloprotease inhibitors.

*Degradation of Bleomycin.* The hydrolysis of a mixture of bleomycins A2 and B2 by bleomycin hydrolase was examined by reversed phase chromatography at 206 nm (Figure 7). The time course shows that bleomycin A2 is

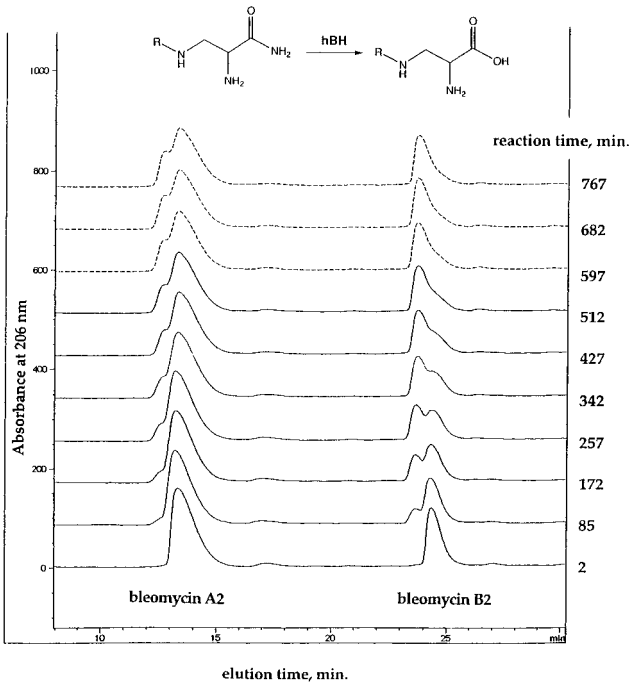


FIGURE 7: Time course of the degradation of bleomycins A2 and B2 by human bleomycin hydrolase as observed by reversed phase chromatography. The enzyme and substrate at pH 7.5, 23 °C, were mixed at time 0, and aliquots from the reaction were injected onto the narrow bore HPLC column every 83 min; the reaction time is listed at the right. Each 206 nm elution profile is stacked immediately above that from the previous injection. Isocratic elution was at 7% acetonitrile−0.1% trifluoroacetic acid. The degradation products of each bleomycin elute (at 0.25 mL/min) slightly in front of the unmodified species. Bleomycin B2 is degraded more rapidly than bleomycin A2. The insert shows the putative hydrolysis in the $\beta$-alanine moiety of bleomycin by hBH.

slowly hydrolyzed to a product which elutes slightly before the reactant, with loss of about a third of bleomycin A2 by 767 min. Bleomycin B2 is degraded more rapidly, with almost complete conversion to a product eluting slightly before the reactant by 600 min. A similar time course was observed at 249 and 292 nm, two other wavelengths with significant absorption by bleomycin (data not shown). The reaction was completely inhibited by preincubation of bleomycin hydrolase with 1 mM iodoacetamide for 10 min before the start of the reaction. The catalytic efficiency ($k_{cat}/K_m$) for the degradation of bleomycin A2 and B2, based on the appearance of the product, at 250 $\mu$M was 5000 $M^{-1}$ $s^{-1}$ and 11 600 $M^{-1}$ $s^{-1}$, respectively.

A plot of the reciprocal of the initial velocity of the reaction vs the reciprocal of the substrate concentration for purified bleomycin A2 and B2 gave apparent $K_m$ values of 140 and 370 $\mu$M, respectively. No evidence was obtained for degradation of bleomycin A2 or B2 by either cathepsin S or cathepsin O2 under several different conditions.

The purified products from the reaction were examined by electrospray mass spectrometry. Bleomycin A2 (expected average $m/z$ = 1415.57, observed = 1414.2 and 1436.0) was degraded by bleomycin hydrolase to give peaks at 1415.2, 1437.0, and 1459.0. These differ by 21.8 and 22.0 amu, respectively, and could represent the binding of two sodium ions with the loss of a proton at each step. The nonadduct product thus appears to be one mass unit more than bleomycin A2 and may bind one more sodium ion.

Bleomycin B2 (expected average $m/z$ = 1425.53, observed = 1425.0 and 1447.2) appears (like bleomycin A2) to bind

a single sodium ion combined with the loss of a proton. Its product gave peaks at *m*/*z* 1426.2, 1448.2, and 1470.0, which could represent the binding of two sodium ions with the loss of a proton at each step. The molecular masses of nonadducted reactant and product appear to differ by a single mass unit, and the product appears capable of binding an additional sodium ion relative to the reactant. The expected mechanism of inactivation of bleomycin by the protease is the deamidation of the $\beta$-aminoalanine residue (Figure 7) which is consistent with the gain of 1 mass unit by the products, and with the creation of an additional sodium ion binding site.

The bleomycin A2 degradation product was also compared to bleomycin A2 by proton nuclear magnetic resonance. The 270 MHz proton spectra of both were nearly identical, the main difference being the loss or shift of a triplet or quartet (ca. 1.6 protons) at 2.88 ppm upon exposure to bleomycin hydrolase. We have not definitively assigned these protons, which do not match those assigned by Xu et al. (1994), but their shift appears closest to that of the $\beta$-aminoalanine $\beta$ or $\beta'$ protons or the pyridinylpropionamide $\alpha'$ proton. One would expect a shift in each of these protons upon deamidation of the nearby terminal amide group.

## DISCUSSION

We have cloned a complete cDNA encoding human bleomycin hydrolase. The open reading frame encodes a 455 amino acid protein which contains the highly conserved active site residues typical for cysteine proteases of the papain family (Figure 1). Sequence alignment with yeast bleomycin hydrolase and with a fragment of the rabbit homologue reveals a 40% and 89.5% identity, respectively (Figure 8). This clearly indicates that the cloned human gene represents the human version of bleomycin hydrolase.

The recombinant human protease has been expressed as an active enzyme in the *Baculovirus* system and acts exclusively as an aminopeptidase. No activity against N-terminal blocked synthetic substrates has been observed. The substrate specificity toward amino acid methyl coumarylamides is relatively broad. The enzyme efficiently hydrolyzes substrates with citrulline, methionine, leucine, alanine, arginine, and glutamate, but it is only weakly active against valine. Substrates with proline, aspartate, and D-alanine in the $P_1$ position are not hydrolyzed. The substrate specificity of the human enzyme is very similar to that of porcine and chicken aminopeptidase H, which are species variants of bleomycin hydrolase (Nishimura et al., 1991; Rhyu et al., 1992; Nishimura et al., 1994). It has been demonstrated that the aminopeptidase activity of the chicken (Rhyu et al., 1992) and yeast homologue (Kamborius et al., 1992) increases with the length of the peptide substrate but is limited to small peptides. However, the enzymes do not hydrolyze protein substrates (Rhyu et al., 1992). The aminopeptidase activity may be explained by the presence of the carboxyl-terminal arm of the protease in the active site cleft, limiting the access to the active site for only one residue. In addition, the carboxyl group of the C-terminal arm may form a salt bridge to the protonated $\alpha$-amino group of the substrate (Joshua-Tor et al., 1995).

Tumor resistance to the anticancer drug bleomycin has been associated with the metabolic degradation of the drug by bleomycin hydrolase (Umezawa et al., 1974; Sebti et al., 1989). It is of interest that most of the tumor cell lines tested

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                                                                      57
hBH   MSSSGLNSEKVAALIQKLNSDPQFVLAQNVGTTHDLLDICLKRATVQRAQHVFQHAV
rBH                        DPQFVLAQNVGTHHDLLDICLRRATVQGAQHVFQHVV
yBH   MSSSIDISKINSWNKEFQSDLTHQLATTVLKNYNADDALLNKTRLQKQDNRVFNTVV
cons  MSSS...S.....................................VF..VV
               *    *                                          114
hBH   PQEGKPITNQKSSGRCWIFSCLNVMRLPFMKKLNIEEPEFSQSYLFFWDKVERCYFF
rBH   PQEGKPVTNQKSSGRCWIFSCLNVMRLPFMKKLNIEEFES-QSYVFFWDKVERCYFF
yBH   STDSTPVTNQKSSGRCWLFAATNQLRLNVLSELNLKEFELSQAYLFFYDKLEKANYF
cons  .....P.TNQKSSGRCW.F...N..RL.....LN..EFE....Y.FF.DK.E....F
                                                                  169
hBH   LSAFVDTAQRKEPEDGRLVQFLLMNPA-NDGGQWDMLVNIVEKYGVIPKKCFPESY-
rBH   LNAFVDTAQKKEPEDGRLVQYLLMNPTQEDGGQWDMLVNIIEKYGVVPKKCFPESH-
yBH   LDQIVSSADQDI--DSRLVQYLLAAPT-EDGGQYSMFLNLVKKYGLIPKDLYGDLPY
cons  L...V..A......D.RLVQ.LL......DGGQ..M..N...KYG..PK........
                                                                  225
hBH   -TTEATRRMNDILNHKMREFCIRLRNLVHSGATKGEISATQDV-MMEEIFRVVCICL
rBH   -TTEASRRMNDILNHKMREFCIRLRNMVHSGATKAEISATQDT-MMEEIFRVVCICL
yBH   STT-ASRKWNSLLTTKLREFAETLRTALKERSADDSIIVTLREQMQREIFRLMSLFM
cons  .TT.ASR..N..L..K.REF...LR..............T....M..EIFR......
                                                                  277
hBH   GNPP----ETFTWEYRDKDKNYQKIGPITPLEFYREHVKPL-FNMEDKICLVNDPRPQ
rBH   GNPP----ETFTWEYRDKDKNYNKIGPITPLEFYRQHVKPLLFNMEDKICFVNDPRPQ
yBH   DIPPVQPNEQFTWEYVDKDKKIHTIKS-TPLEFASKYAKLDPSTP---VSLINDPR--
cons  ..PP....E.FTWEY.DKDK....I...TPLEF.....K.............NDPR..
                                                                  334
hBH   HKYNKLYTVEYLSNMVGGRKTLYNNQPIDFLKKMVAASIKDGEAVWFGCDVGKHFNS
rBH   HKYNRLYTVDYLSNMVGGRK
yBH   HPYGKLIKIDRLGNVLGGDAVIYLNVDNETLSKLVVKRLQNNKAVFFGSHTPKFMDK
cons  H.Y..L.....L.N..GG....Y.N.....L...V........AV..G....K....
                                              *                   391
hBH   KLGLSDMNLYDHELVFGVSLKNMNKAERLTFGESLMTHAMTFTAVSEKDDQDGAFTK
yBH   KTGVMDIELWNYPAIGYNLPQQKASRIRYH--ESLMTHAMLITGCHV-DETSKLPLR
cons  K.G..D..L..................R....ESLMTHAM..T.....D........
            *                                                 I 445
hBH   WRVENSWGEDHGHKGYLCMTDEWFSEYVYEVVVDRKHVPEEVLAVL---EQEPIVLP
yBH   YRVENSWGKDSGKDGLYVMTQKYFEEYCFQIVVDINELPKELASKFTSGKEEPIVLP
cons  .RVENSWG.D.G..G...MT...F.E.....VVD....P.E..........EPIVLP
                455
hBH   AWDPMGALAE
yBH   IWDPMGALAK
cons  .WDPMGALA.
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FIGURE 8: Multiple amino acid alignment of human bleomycin hydrolase with yeast and a partial rabbit sequence of bleomycin hydrolase. *, active site residues. Numbers in the right margin indicate the position of the last amino acid in each line.

display relatively higher expression levels than seen in regular human organs. Especially high levels were observed in leukemia cell lines. Leukemic cancer is known not to respond to bleomycin treatment. Specific inhibitiors of the enzyme in tumors may (i) widen the application of bleomycin to tumors which are resistant to a bleomycin treatment and (ii) allow a lower dose in order to diminish observed side effects, especially pulmonary toxicity. The present study demonstrates for the first time the *in vitro* ability of human bleomycin hydrolase to degrade bleomycin. The human enzyme attacks bleomycin B2 approximately 2 times more rapidly than A2. Our results are consistent with catabolism of both bleomycins A2 and B2, resulting in a shift in mass

of 1 amu and slightly earlier elution from a reversed phase column. This could be due to deamidation of a terminal amide group, which is consistent with the proton NMR data. These observations are also consistent with those of Umezawa et al. (1972) that bleomycin hydrolase deamidates the carboxamide group of the $\beta$-aminoalanine moiety.

E-64 is frequently used in *in vitro* experiments to decrease the resistance of tumor cells against bleomycin. Our data show that the human homologue of bleomycin hydrolase is inhibited by E-64 but relatively high doses ($>50$ $\mu$M) are needed for inhibition. The second order rate constant of inactivation is only 60 $M^{-1}$ $s^{-1}$ and at least 3 orders of magnitude lower than those observed for related papain-like cathepsins (Gour-Salin et al., 1994). Although species variants of bleomycin hydrolase reveal some differences in their inhibition with E-64, there is presently no efficient inhibitor of the protease known which is specific and potent enough to justify animal studies.

The biological function of bleomycin hydrolase remains unclear. Its ubiquitous distribution and evolutionary conservation from bacteria to human may indicate an important physiological role. The recently published X-ray structure describes bleomycin hydrolase as a 3-symmetrical hexamer. The active sites of the six monomers are buried in a channel in the center of the hexamer and are probably accessible only to small peptides. The human peptidase may be similarly structured since it is also a hexamer of six identical subunits. The structural organization of yeast bleomycin hydrolase resembles strikingly that of a proteasome (Joshua-Tor et al., 1995). Since bleomycin hydrolase seems to be localized in the cytosol/nuclear compartment, exhibits a neutral to slightly alkaline pH optimum, is very efficient in the hydrolysis of small peptides, and displays a similar ubiquitous tissue distribution as the proteasome, a concerted action of both enzymes may be imagined. A major biological function of bleomycin hydrolase could be the final degradation of peptides released from the proteasome.

Besides its function as an aminopeptidase, a protease independent role of bleomycin hydrolase has been postulated. Xu and Johnston (1994) have shown that the yeast bleomycin hydrolase binds to a specific DNA sequence corresponding to the upstream activating sequences of GAL4-inducible genes, suggesting that yBH may also have a role in gene regulation. However, expression in a variety of yeast genetic backgrounds of either cloned yBH or fusion proteins, linking yBH to the activation domain of the GAL4 protein, have failed to show any influence on transcriptional activity at GAL4-dependent promoter sites in our lab (S. P. Smeekens, unpublished). Joshua-Tor et al. (1995) suggest that the prominent positive electrostatic potential inside the channel of the hexamer structure of yBH may represent the region where the DNA binds to the aminopeptidase. The electrostatic potential in the yeast enzyme is formed by 12 lysine residues/subunit. However, seven of these residues are replaced by neutral or negatively charged residues in the human sequence. In particular, lysine residue 245 which is located at the outer rim of the channel of the yeast structure and which is described as essential for the binding of single-stranded DNA is replaced by a tyrosine in hBH. Thus binding to DNA by the yeast bleomycin hydrolase might be a specific feature of this species variant.

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