Human tumour cathepsin B

Comparison with normal liver cathepsin B

Kamiar MOIN,* Nancy A. DAY,* Mansoureh SAMENI,* Sadiq HASNAIN,†
Tomoko HIRAMA† and Bonnie F. SLOANE*‡

*Department of Pharmacology Wayne State University, Detroit, MI 48201, U.S.A.,
and †Institute for Biological Sciences, National Research Council, Ottawa, Ontario K1A 0R6, Canada

Cathepsin B was purified from normal human liver and several human tumour tissues and partially characterized. Three forms of cathepsin B, with molecular masses of 25 kDa, 26 kDa (the two appearing as a doublet) and 30 kDa, were detected in SDS/polyacrylamide gels. The 25–26 kDa doublet was associated with the fractions from tumours and normal liver containing the highest cathepsin B activity. Cathepsin B from both sources showed similar pH optima. Both normal liver and tumour cathepsin B exhibited similar kinetics against selected synthetic substrates. At neutral pH and 24 °C, cathepsin B from both normal liver and tumour exhibited a lower K_m and a higher k_{cat}/K_m than at pH 6.0. Their inhibitory profiles against synthetic inhibitors were also similar. Immunological studies with a monospecific antibody against the mature double-chain form of human liver cathepsin B and an antibody against a cathepsin B-derived synthetic peptide established the immunological similarity of liver and tumour enzymes. The *N*-terminal sequences of the 25 kDa and 26 kDa forms were identical with that of the heavy chain of the mature double-chain form of human cathepsin B, whereas the *N*-terminal sequence of the 30 kDa species was identical with that of the single-chain form of human cathepsin B. Treatment of the double-chain form of cathepsin B from normal liver and tumours with the endoglycosidase peptide- N^4 -(*N*-acetyl- β -glucosaminyl)asparagine amidase converted the 26 kDa form into 25 kDa in SDS/polyacrylamide gels, suggesting that cathepsin B may exist as both glycosylated and unglycosylated forms. Our results, in contrast with those reported earlier for mouse cathepsin B, indicate that human liver and tumour cathepsin B are similar.

INTRODUCTION

Intravasation of cancer cells into the circulatory and/or the lymphatic systems and their extravasation out of the vasculature into host parenchymal tissue are necessary prerequisites to successful metastasis. During intravasation and extravasation there is dissolution of the basement membrane by proteinases. The cysteine endopeptidase cathepsin B is one of the proteolytic enzymes that has been implicated in tumour invasion and metastasis (for reviews, see [1–3]).

Cathepsin B is a lysosomal enzyme believed to participate in the intracellular degradation and turnover of proteins [4]. Mature cathepsin B exists in two forms, a single-chain form and a double-chain form [5,6]. Mature single-chain cathepsin B consists of 254 amino acids and is ~30 kDa in size [6]. The double-chain form consists of a heavy chain of ~25 kDa and a light chain of ~5 kDa [5,6]. The double-chain form is derived from the single-chain form as a result of enzymic cleavages between residues 47 and 50 with the loss of a dipeptide [6].

Lysosomal enzymes are directed to the lysosomes via a receptor-mediated system with mannose 6-phosphate as the recognition marker [7,8]. On the basis of the ability of mannose 6-phosphate to inhibit endocytosis of secreted procathepsin B cathepsin B is thought to be trafficked via mannose phosphate receptors [9]. In I-cell fibroblasts, lysosomal enzymes are secreted as a result of the defective phosphorylation of the mannose 6-phosphate recognition marker [10–12]. Cathepsin B is secreted from human and murine tumours [13–16], but, in contrast with the situation in I-cell disease [11], not all lysosomal enzymes are secreted [14]. In I-cell fibroblasts, small vesicles containing lysosomal enzymes are observed near the cell periphery [12].

Cytochemical and immunochemical studies on tumour cells indicate a similar peripheral distribution of cathepsin B activity and protein [17–19]. This presence of cathepsin B in small vesicles near the periphery of tumour cells may be responsible for the co-sedimentation of cathepsin B with plasma membrane/endosomal fractions of tumour cells, a phenomenon we have observed in all malignant cells of murine and human origin (for reviews, see [1–3,20]).

This altered distribution of cathepsin B in tumours may indicate changes at one or more steps in the normal pathway for biosynthesis and delivery of cathepsin B to lysosomes (i.e., alterations in transcription/translation, co- and post-translational processing and/or in intracellular trafficking). In the present study we purified and characterized cathepsin B from human tumours in order to evaluate possible changes in cathepsin B itself.

MATERIALS AND METHODS

Materials

L-trans-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64), dithiothreitol (DTT), leupeptin, aprotinin and pepstatin A were purchased from Sigma (St. Louis, MO, U.S.A.). benzyloxycarbonyl-Phe-Arg *p*-nitroanilide (Z-Phe-Arg-*p*NA) and Z-Arg-Arg-*p*NA were from Bachem U.S. (Philadelphia, PA, U.S.A.). benzyloxycarbonyl-Arg-Arg *N*-methylcoumarylamide (Z-Arg-Arg-NHMec), Z-Phe-Arg-NHMec, Sephadex-aminoethyl-Gly-Phe-Gly semicarbazone (Sephadex-Ahx-Gly-Phe-Gly-SC), and Z-Phe-Ala-CHN₂ were obtained from Enzyme System Products (Livermore, CA, U.S.A.). Nitrocellulose membranes were purchased from Bio-Rad (Richmond, CA, U.S.A.). Immobilon-P

Abbreviations used: Con A, concanavalin A; E-64, *L-trans*-epoxy succinyl-leucylamido(4-guanidino)butane; Endo F, endo- β -*N*-acetylglucosaminidase F; PNGase F, peptide- N^2 -(*N*-acetyl- β -glucosaminyl)asparagine amidase; Z, benzyloxycarbonyl; NHMec, *N*-methylcoumartin; -CHN₂, -diazomethane; DTT, dithiothreitol; -*p*NA, *p*-nitroanilide; Ahx, aminoethyl; -SC, semicarbazone; Aan, α -aminoacetoneitrile.

‡ To whom all correspondence should be addressed.

[poly(vinylidene difluoride)] membranes were from Millipore Corp. (Bedford, MA, U.S.A.). Endo- β -*N*-acetylglucosaminidase (Endo F) and peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase (PNGase F) were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other chemicals were of analytical grade.

Methods

Tumours. Normal human liver tissue was obtained from autopsy and frozen at -20°C before use. Tumour samples (human breast carcinoma, colon carcinoma, melanoma, ovarian carcinoma and sarcoma) were obtained from surgical resections and quickly frozen at -70°C . Tumours were transferred to our laboratory and stored at -20°C before use. Tumours of the same histological classification were pooled to provide sufficient amounts of starting material for purification.

Purification of cathepsin B. Cathepsin B was purified from normal human liver and human tumours by a modification of the procedures of Barrett [21] and Rich *et al.* [22]. Frozen liver or tumour tissue was partially thawed, minced and homogenized in a Cuisinart Food Processor (Greenwich, CT, U.S.A.) in two parts (w/v) of an ice-cold solution of 1% (w/v) NaCl, 2% (v/v) butanol-1-ol and 1 mM-disodium EDTA. Triton X-100 was added to give a final concentration of 0.2% (v/v), and the pH was adjusted to 4.4 with 2.5 M-sodium formate/formic acid buffer, pH 2.8. The homogenate was stirred for 4 h at 37°C , overnight at 4°C , and centrifuged at 17000 *g* for 15 min.

Acetone (0.5 vol) prechilled to -10°C was added to the clear supernatant, with stirring, over a period of 10 min. The first precipitate was removed by centrifugation at 2500 *g* for 15 min and discarded. A further portion of acetone (0.9 vol) was cooled and added to the supernatant as described above. The second precipitate, containing cathepsin B, was collected by centrifugation at 17000 *g* for 15 min and resuspended in a minimum volume of 50 mM-sodium phosphate/1 mM-EDTA, pH 6.0 (buffer A). The suspension was stirred for 2 h and centrifuged at 27000 *g* for 15 min to remove insoluble material. The supernatant was filtered through Whatman no. 1 filter paper and the filtrate dialysed overnight against 4 litres of buffer A. The dialysis residue was centrifuged at 27000 *g* for 15 min and then concentrated to 3–5 ml in an Amicon model 40L concentrator (Danvers, MA, U.S.A.) fitted with a YM-10 membrane.

The affinity resin Sephadex-Ahx-Gly-Phe-Gly-SC (5 ml) was washed twice with 0.1 M-Tris, pH 8.0, and equilibrated first with 50 mM-sodium formate/1 mM-EDTA, pH 4.0, and then with buffer A. DTT was added to the concentrated sample at a final concentration of 2 mM. The sample was mixed with the affinity resin at room temperature for 2 h. The resulting slurry was placed in a column (1.0 cm $\times$ 7.0 cm) and the unbound fractions collected. The column was subsequently washed with 50 ml of buffer A containing 0.5 M-NaCl at a flow rate of 1 ml/min, followed by 50 mM-sodium formate, pH 4. Cathepsin B was eluted with 50 mM-sodium formate, pH 4.0, containing 1.5 mM-2,2-dipyridyl disulphide and concentrated to a volume of 3–5 ml as described above.

The concentrated sample from the affinity column was dialysed in 50 mM-sodium acetate/1 mM EDTA, pH 4.2, at 4°C overnight and applied to a Mono S f.p.l.c. column (Pharmacia-LKB, Piscataway, NJ, U.S.A.). Cathepsin B was eluted from the column with a linear gradient of NaCl (0–0.5 M) in 50 mM-sodium acetate/1 mM-EDTA, pH 4.2, and 0.5 ml fractions were collected.

SDS/PAGE. Electrophoresis was performed in 12%-(w/v)-polyacrylamide gels in the presence of SDS in the buffer system described by Laemmli [23]. Gels were silver stained according to

the method of Merril *et al.* [24]. The molecular mass of cathepsin B was determined by comparing the mobility of the purified enzyme in polyacrylamide gels with those of the standards (bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; lactalbumin, 14 kDa). The results are expressed as the logarithm of the molecular mass versus relative mobility.

Development of monospecific antibodies. Antisera against native human liver cathepsin B and a cathepsin B-derived synthetic peptide, Gly-Tyr-Ser-Pro-Thr-Tyr-Lys-Gln-Asn-Lys-His-Tyr-Gly (residues 135–147; see [6]), were raised in rabbits (New Zealand White, male). An equal volume of a 0.15 M-NaCl solution was added to the collected serum sample and the mixture saturated to 50% with $(\text{NH}_4)_2\text{SO}_4$. The suspension was then stirred at room temperature for 45–60 min and centrifuged at 12000 *g* for 10 min at 4°C . The pellet was dissolved in distilled water and the resulting solution equilibrated with 20 mM-sodium phosphate buffer, pH 6.4, using a PD-10 column (Pharmacia-LKB). Non-IgG proteins were removed by applying the serum sample to a DEAE-Affigel Blue column (Bio-Rad) equilibrated with the same buffer. The unbound fraction containing IgG was collected, concentrated and NaCl was added to a final concentration of 0.15 M. An IgG fraction was purified using Protein A-Superose (Pharmacia-LKB) that had been equilibrated with 1.5 M-glycine/3 M-NaCl, pH 8.9. The IgG was eluted with 100 mM-citric acid, pH 4.0, concentrated and dialysed against 20 mM-potassium phosphate buffer, pH 7.2, containing 0.015 M-NaCl and stored at -20°C . The specificity of the above antibodies was tested against normal human liver and human tumour cathepsin B, as well as normal human cathepsin L (a gift from Dr. Vito Turk, Josef Stefan Institute, Ljubljana, Slovenia) in a Minifold II slot-blot apparatus (Schleicher and Schuell, Keene, NH, U.S.A.) according to the manufacturer's instructions.

Immunoblot analysis. Electrophoresed antigen in 12%-(w/v)-polyacrylamide-gel slabs was transferred to nitrocellulose membranes (Bio-Rad) by using a Hoefer Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) and 25 mM-Tris/200 mM-glycine/20% (v/v) methanol, pH 8.3, as the transfer buffer. When transfer was complete (30 V overnight and 70 V for 1 h), membranes were developed utilizing the purified antibodies described above and horseradish peroxidase according to the Bio-Rad procedure. Alternatively, the membranes were developed with the more sensitive ECL Western-blot detection system (Amersham, Arlington Heights, IL, U.S.A.). Dry milk (5%) and Tween were used as blocking agents for both methodologies.

Peptide sequencing. For *N*-terminal sequencing, samples containing purified cathepsin B were subjected to SDS/PAGE (see above), except that the running buffer contained 0.1 M-sodium thioglycollate in the upper chamber and the SDS was recrystallized as described by Hunkapiller *et al.* [25]. The gel was transferred to Immobilon-P membrane and the membrane stained with Coomassie Brilliant Blue according to the manufacturer's (Millipore) instructions. Direct protein microsequencing from the Immobilon-P was performed by Dr. Joseph Leykam of the Macromolecular Structure Facility of Michigan State University (East Lansing, MI, U.S.A.).

Carbohydrate detection. To detect the presence of carbohydrate moieties, the purified double-chain forms of cathepsin B from both normal human liver and human tumour tissues were incubated with Endo F and/or PNGase F as described previously [26,27]. The samples were then subjected to SDS/PAGE and the gels silver-stained for analysis. In addition, double-chain cathepsin B from tumour was applied to a 3 ml concanavalin A

(Con A)-Sephacose column equilibrated with 0.02 M-Tris/HCl/0.5 M-NaCl, pH 7.4, and the unbound fractions collected. Bound cathepsin B was eluted in 20–30 ml of the equilibration buffer containing 0.5 M- α -D-mannose. Unbound fractions and eluted fractions were then concentrated to 300 μ l and the buffer exchanged to 50 mM-sodium acetate/1 mM-EDTA, pH 5.2, utilizing a Centricon-10 microconcentrator (Amicon, Beverly, MA, U.S.A.). Portions of each sample were analysed by immunoblot analysis as described above.

Cathepsin B assays. Stopped assays. The enzyme was activated with 5 mM-DTT and 2.5 mM-EDTA at pH 5.2 for 15 min at 37 °C. The assay was performed by the addition of citrate phosphate buffer (final concn. 0.1 M), pH 6.2, and Z-Arg-Arg-NHMec as substrate (final concn. 5 μ M). The reaction was stopped with monochloroacetate, pH 4.3, after 5, 15, and 25 min of incubation. Relative fluorescence was measured in a Shimadzu RF-540 spectrofluorophotometer at an excitation of wavelength 370 nm and an emission wavelength of 460 nm. The stopped assays described above were used for the determination of activity in large numbers of samples (e.g. during purification). One unit of enzyme activity is defined as the amount of enzyme required to release 1 μ mol of NHMec/min.

Continuous assays. Continuous rate assays using Z-Arg-Arg-pNA and/or Z-Phe-Arg-pNA as substrate were used for kinetic analysis. In this case the activation step was performed with 10 mM-DTT and 0.05 % Brij-35, pH 6.0, for 1 h at room temperature. The reaction buffer was 25 mM-sodium phosphate/1 mM-EDTA/250 mM-NaCl, pH 6.0. Substrate concentration was varied from 0.025 mM to 0.15 mM (Z-Phe-Arg-pNA) and from 0.1 to 2.3 mM (Z-Arg-Arg-pNA); dimethyl sulphoxide was added to a final concentration of 3 %. Activated enzyme in a small volume [final concn. 4.6 nM as determined by titration against E-64 (see below)] was added and the reaction was continuously monitored at 380 nm (ϵ 13070 M⁻¹·cm⁻¹) or 410 nm (ϵ 8800 M⁻¹·cm⁻¹) in a SLM Aminco DW2000 spectrophotometer at 24 °C. Initial rates were calculated from the linear portion of the recorded tracing. Values for K_m were calculated from Hanes–Woelf plots obtained by plotting [S]/ v against [S] (not shown), where [S] and v are substrate concentration and rate of reaction respectively. Data were fitted by a non-linear-regression data-analysis program, ENZFITTER of Leatherbarrow, supplied by Elsevier-Biosoft. k_{cat} was calculated from the ratio $V_{max}/[E_0]$ where $[E_0]$ is the initial enzyme concentration. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μ mol of *p*-nitroaniline/min.

Active-site titration. The molar concentration of cathepsin B was measured by titration against E-64. Enzyme was preincubated in the presence of 10 mM-DTT/0.05 % Brij-35, pH 6.0, and E-64 in concentrations ranging from 0 to 1.7×10^{-7} M for 1 h at room temperature. The inhibitory activity against Z-Phe-Arg-NHMec (final concn. 24 μ M) was monitored at an excitation wavelength of 370 nm and an emission wavelength of 460 nm as described above under 'Stopped assays'.

Inhibitor studies. The enzymes were assayed in the presence of various inhibitors as described above under 'Stopped assays'. The inhibitor was added to the mixture after the addition of the assay buffer and incubated for 5 min before the addition of the substrate. The percentage inhibition was determined by comparing the activities with those measured for positive controls without inhibitors. The apparent K_i was calculated utilizing the fractional-inhibition formula for competitive inhibition at a single concentration of the inhibitor as described by Nicklin & Barrett [28].

Protein determination. The Micro BCA Kit (Pierce, Rockford, IL, U.S.A.) was utilized to determine protein concentrations; the procedure described by the manufacturer was followed.

RESULTS

Purification of cathepsin B

Cathepsin B was purified from normal human liver and human tumour tissues by acid fractionation, acetone fractionation, affinity chromatography and cation-exchange chromatography. Table 1 shows a purification of cathepsin B from human sarcoma. Rich *et al.* [22] had reported a purification factor of 361 for human liver cathepsin B as compared with our 673-fold purification after the affinity column. Our calculations are based on the homogenate as the starting point, whereas those of Rich *et al.* [22] were based on the post-autolysis (acid-fractionation) step. When our fold purification was calculated from the acid-fractionation step, it was 224-fold or somewhat less than that reported by Rich *et al.* [22]. This lesser fold purification may be due to overloading of the affinity column, as our yield was considerably less than that reported by Rich *et al.* [22]. In order to separate a contaminant 36 kDa protein, we added a final cation-exchange-chromatography step. The *N*-terminal sequence of the protein running at 36 kDa differed from that of any known cysteine endopeptidase (as determined by computer analysis against DNA sequences in the GenBank; B. A. Frosch, N. A. Day, K. Moin, S. Hasnain and B. F. Sloane, unpublished work).

Single peaks of cathepsin B activity were detected in elution profiles from the mono S column for both human liver and tumour samples (results not shown). Maximum cathepsin B activity from both normal liver and tumour tissues was eluted in the same fractions at ~ 0.25 M-NaCl, suggesting that normal and tumour cathepsin B have similar biochemical characteristics. The purified cathepsin B was ≥ 90 % active as determined by E-64 titration (results not shown).

Determination of molecular masses

The molecular masses of cathepsin B from tumour and normal liver tissues were estimated on SDS/polyacrylamide gels under reducing conditions. Samples corresponding to individual fractions with cathepsin B activity from the mono S column were applied to 12 %-polyacrylamide gels and electrophoresis performed as described in the Materials and methods section. Three distinct bands of molecular masses 30 kDa, 26 kDa and 25 kDa (the last two appearing as a doublet) were observed in fractions from both normal human liver and tumour tissues (Fig. 1). These probably represent the single-chain form of cathepsin B and a heavy-chain doublet of the double-chain form of cathepsin B respectively [5,6]. Although we were able to obtain fractions containing only the double-chain forms of cathepsin B, we were unable to obtain a homogeneous preparation containing only the single-chain form of cathepsin B. The fractions from the mono S column with the highest levels of cathepsin B activity corresponded to those containing the 25–26 kDa doublet on SDS/PAGE. The binding of the double-chain forms of cathepsin B to mono S differed, as indicated by their presence in different fractions from the mono S column (results not shown), suggesting that the two differ in charge (see under 'Carbohydrate analysis' below).

Immunoblot analysis

Antibodies were raised against the double-chain form of human liver cathepsin B and a synthetic peptide (see the Materials and methods section). The synthetic peptide, unique to cathepsin B (as determined by computer analysis against DNA sequences in the GenBank), corresponded to residues 135–147 [6]:

Table 1. Purification of cathepsin B from human sarcoma

Purification stage	Volume (ml)	[Protein] (mg/ml)	Total Protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Homogenate*	414	21	8715	0.02	7.2	0.001	100	1
Acid	305	6.2	1891	0.013	4	0.002	56	3
Acetone	48	8	383	0.2	9.4	0.025	130	30
Affinity	5.7	0.25	1.4	0.14	0.8	0.55	11	673
Mono S	2.0	0.25	0.5	0.44	0.9	1.8	12.3	2146

* A 138 g portion of human sarcoma was used.

Fig. 1. SDS/PAGE of purified cathepsin B

Approx. 5 μ g of each sample was loaded and the gels silver-stained for analysis. (a) Normal human liver; (b) human sarcoma. Lane numbers correspond to fractions from the mono S column. S, molecular-mass (M) standards.

Fig. 2. Immunoblot (a and b) and SDS/PAGE (c) analysis of samples from different stages of purification of cathepsin B from normal human liver and human sarcoma

Samples from human liver (a, < 1 μ g/lane) and human sarcoma (b and c, 5 μ g/lane) were electrophoresed in 12%-polyacrylamide gels and the gels silver-stained or electrophoretically transferred to nitrocellulose membranes. The membranes were developed utilizing an antibody to human liver cathepsin B (see the Materials and methods section). Lanes represent: 1, acetone fraction; 2, pooled affinity fraction; 3, mono S fraction with highest cathepsin B activity; 4, molecular-mass (M) standards.

The sequence is common to both single-chain cathepsin B and the heavy chain of double-chain cathepsin B. The specificity of the antibodies was confirmed by screening against the purified double-chain form of human liver cathepsin B and cathepsin L (a related cysteine endopeptidase) by dot-blotting. The antibodies did not recognize cathepsin L, but reacted strongly with cathepsin

B (results not shown). Since this technique does not allow one to detect different forms of cathepsin B, we analysed normal liver and tumour cathepsin B by immunoblotting. In the samples from human liver and tumour, strong reactions were detected against the 25, the 26 and the 30 kDa bands with both antibodies (Figs. 2 and 3). The specificity of our antibodies was apparent, since

Fig. 3. SDS/PAGE (a) and immunoblot analysis (b) of mono S fractions of human sarcoma that contain cathepsin B activity

In (b) the antibody specific to the cathepsin B-derived synthetic peptide was utilized to confirm the identity of the bands as cathepsin B. Lane numbers ('84' and '93') correspond to fractions from the mono S column. Molecular masses (*M*) are indicated at the left.

they reacted with only the cathepsin B bands in the acetone and affinity fractions (Figs. 2a and b, lanes 1 and 2). No cross-reactivity was observed with the molecular-mass standards (Figs. 2a and b, lane 4). Cathepsin B from both normal and tumour tissues was immunologically similar.

N-Terminal analysis

Electrophoresed cathepsin B samples from human sarcoma containing single- and double-chain forms were transferred to Immobilon nylon membranes and the membranes stained with Coomassie Brilliant Blue for direct microsequencing (see Materials and methods section). The *N*-terminal sequence of the 30 kDa band was identical (Fig. 4) to the *N*-terminal sequence of the single-chain form of human cathepsin B [6]. The 25 and the 26 kDa bands of the doublet had identical *N*-terminal sequences, both of which corresponded to the *N*-terminus of the heavy chain of the double-chain form of cathepsin B (Fig. 4). Thus the double-chain form of cathepsin B exists as multiple isoforms.

Carbohydrate analysis

Since the 25 and the 26 kDa bands had identical *N*-terminal

Fig. 5. Removal of oligosaccharide moieties from cathepsin B

The mature double-chain form of cathepsin B from normal human liver and human sarcoma was incubated with PNGase F (see the Materials and methods section) and analysed by SDS/PAGE. α_1 -Acid glycoprotein was used as positive control. Lanes 1, 3 and 5 contained samples incubated without PNGase F. Lanes 2, 4 and 6 contained samples incubated with PNGase F. Lanes represent: 1 and 2, human liver cathepsin B; 3 and 4, human sarcoma cathepsin B; 5 and 6, α_1 -acid glycoprotein; 7, molecular-mass (*M*) markers.

sequences (Fig. 4) and reacted with both anti-(cathepsin B protein) and anti-(cathepsin B peptide) antibodies (Figs. 2 and 3), we considered the possibility that the difference in their molecular mass was due to smaller, or even the absence of, carbohydrate moieties on the 25 kDa heavy chain. Therefore we treated double-chain cathepsin B from both liver and sarcoma with endoglycosidase F and PNGase F (see the Materials and methods section). The mobility of the 25 kDa and the 26 kDa bands was not affected by incubation with endoglycosidase F (results not shown), demonstrating that cathepsin B oligosaccharides were not of the complex type. By contrast, after incubation with PNGase F, the 26 kDa band co-migrated with the 25 kDa band (Fig. 5), indicating that the 26 kDa heavy chain has an *N*-linked high-mannose oligosaccharide.

A sample containing the double-chain form of cathepsin B was subjected to Con A-Sepharose chromatography, and the unbound fractions and the fractions eluted with α -D-mannose

Single-chain form		
<i>N</i> -Terminal sequence of	1	12
human liver cathepsin B:	Leu-Pro-Ala-Ser-Phe-Asp-Ala-Arg-Glu-Gln-Trp-Pro	
<i>N</i> -Terminal sequence of		
30 kDa band from sarcoma:	Leu-Pro-Ala-Ser-Phe-Asp-Ala-Arg-Glu-Gln-Xaa-Pro	
Heavy chain of double-chain form		
<i>N</i> -Terminal sequence of	50	60
human liver cathepsin B:	Val-Ser-Val-Glu-Val-Ser-Ala-Glu-Asp-Leu-Leu	
<i>N</i> -Terminal sequence of		
upper band of 25-26 kDa		
doublet from sarcoma:	Val-Ser-Val-Glu-Val-Ser-Ala-Glu-Asp-Leu-Leu	
<i>N</i> -Terminal sequence of		
lower band of 25-26 kDa		
doublet from sarcoma:	Val-Ser-Val-Glu-Val-Ser-Ala-Glu-Asp-Leu	

Fig. 4. Comparison of the *N*-terminal sequences of the 30, 26 and 25 kDa bands purified from human sarcoma with the *N*-terminal sequences of single-chain human liver cathepsin B and of the heavy chain of double-chain human liver cathepsin B [6]

Fig. 6. Interaction of the double-chain form of mature human sarcoma cathepsin B with Con A

Cathepsin B was applied to a Con A-Sepharose column, and the column was eluted with α -D-mannose. Lanes represent: 1, unbound fraction; 2, bound fraction. Arrows depict 25 and 26 kDa forms.

analysed by immunoblotting. The unbound fractions contained both the 25 kDa and the 26 kDa bands (Fig. 6). This is not surprising, since the carbohydrate structures determined for pig and rat cathepsin B [29,30] suggest that binding to Con A will be with low affinity. However, as shown in Fig. 6, a small amount of cathepsin B did bind to Con A-Sepharose and was eluted with α -D-mannose. The mobility of the eluted cathepsin B in SDS/polyacrylamide gels corresponded to 26 kDa (Fig. 6). Since only the double-chain form with the 26 kDa heavy chain was eluted from the Con A-Sepharose column, we concluded that the 25 kDa heavy chain of cathepsin B lacks an oligosaccharide chain with sufficient terminal mannose residues to bind to Con A.

pH studies

Cathepsin B from normal liver and tumours was assayed at various pH values to determine its pH optimum. Cathepsin B from both liver and tumour tissues appeared to have optimal activity from pH 5.5 to 7.5 (results not shown).

Determination of kinetic constants

Kinetic constants for the hydrolysis of synthetic substrates by cathepsin B from normal human liver and human sarcoma and synthetic substrates are summarized in Table 2. We tested cathepsin B from human sarcoma because this tumour could be obtained in amounts sufficient for the purification of the quantities of cathepsin B required for kinetic analysis. A comparison of the kinetic constants for the hydrolysis of Z-Phe-Arg-pNA at pH 6.0 and for Z-Arg-Arg-pNA at pH 6.0 and 7.4 (Table 2) indicated that the human sarcoma enzyme was catalytically identical with normal human liver cathepsin B under the conditions of the present studies. The $k_{\text{cat.}}/K_m$ of both liver and

Table 3. Inhibition of human liver and sarcoma cathepsin B by peptide inhibitors

Values are means $\pm$ S.D.

Inhibitor (70 μ M)	Liver cathepsin B		Sarcoma cathepsin B	
	$K_{i,\text{app.}}$ (μ M)	Inhibition (%)	$K_{i,\text{app.}}$ (μ M)	Inhibition (%)
None	—	0	—	0
Z-Gly-Phe-Aan	43.8 ± 6.5	62	63.6 ± 2.6	53
Z-Gly-Phe-Gly-SC	6.0 ± 0.7	92	7.1 ± 0.8	91
Z-Gly-Phe-Phe-SC	3.2 ± 0.5	96	2.7 ± 0.3	96
Z-Phe-Gly-SC	4.1 ± 0.7	99	3.5 ± 0.6	96
Z-Phe-Phe-SC	3.5 ± 0.5	95	2.5 ± 0.3	96

tumour cathepsin B was 4-fold higher for Z-Phe-Arg-pNA than for Z-Arg-Arg-pNA (Table 2). These results confirm the previous finding that synthetic substrates containing a blocked Phe-Arg dipeptide are preferred by cathepsin B [4]. Nevertheless, as cathepsin L also reacts strongly with a blocked Phe-Arg dipeptide [4], Z-Phe-Arg-pNA is not the substrate of choice for cathepsin B in unpurified samples. Against Z-Arg-Arg-pNA an approx. 2-fold increase in the $k_{\text{cat.}}/K_m$ was observed for both liver and tumour cathepsin B at pH 7.4, as compared with pH 6.0 (Table 2). This increase in $k_{\text{cat.}}/K_m$ was primarily due to a decrease in the K_m .

Inhibitor studies

Human tumour cathepsin B was inhibited $\geq 99\%$ by three cysteine endopeptidase inhibitors tested [leupeptin (10 μ M), Z-Phe-Ala-CHN₂ (5 μ M), and E-64 (10 μ M)], but only inhibited $\leq 2\%$ by pepstatin (1 μ M), an aspartic-endopeptidase inhibitor, or aprotinin (400 kallikrein-inhibitory units), a serine-endopeptidase inhibitor (results not shown). Similar inhibitor profiles have been established for human liver cathepsin B [4] and were confirmed in our laboratory against human liver cathepsin B (results not shown). We also tested a series of synthetic peptides, Z-Gly-Phe-Aan (Aan = α -aminoacetonitrile), Z-Gly-Phe-Gly-SC, Z-Gly-Phe-Phe-SC, Z-Phe-Gly-SC, Z-Phe-Phe-SC, designed as inhibitors of cysteine endopeptidases by Dr. D. Rich (University of Wisconsin, Madison, WI, U.S.A.). No differences were observed in their abilities to inhibit cathepsin B from either tumour or liver (Table 3).

DISCUSSION

The association of cathepsin B activity with plasma membrane/endosomal fractions is the most striking difference between tumours and normal tissues (for reviews, see [1–3,20]). The nature of this association has not been determined. However, the present study suggests that cathepsin B in malignant tumours does not have major structural modifications. Human cathepsin B from normal liver and tumours were similar in respect of their molecular masses. N-terminal sequences, antibody reactivities,

Table 2. Kinetic parameters for the hydrolysis of synthetic dipeptide substrates by human liver and sarcoma cathepsin B

Values are means $\pm$ S.D.

Parameter	Z-Phe-Arg-pNA (pH 6.0)		Z-Arg-Arg-pNA (pH 6.0)		Z-Arg-Arg-pNA (pH 7.4)	
	Liver	Sarcoma	Liver	Sarcoma	Liver	Sarcoma
$k_{\text{cat.}}$ (s^{-1})	33.8 ± 5.06	37.0 ± 5.7	12.8 ± 0.4	12.1 ± 0.8	21.8 ± 2.8	20.3 ± 2.6
K_m (mM)	0.6 ± 0.10	0.7 ± 0.06	0.9 ± 0.06	0.9 ± 0.10	0.7 ± 0.03	0.7 ± 0.05
$k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	54600 ± 1160	53400 ± 8230	14800 ± 690	13300 ± 771	32190 ± 2810	28730 ± 2473

pH optima, kinetic parameters and inhibition profiles. Our results conflict with those in an earlier study by Olstein & Liener [31] on cathepsin B and an 'endogenous thiol proteinase' from murine liver and a chemically induced murine sarcoma. To our knowledge, this study by Olstein & Liener [31] is the only comparative report on cathepsin B from a normal tissue and its tumour analogue. On the basis of amino acid composition, peptide mapping, molecular masses and kinetic parameters, Olstein & Liener [31] concluded that cathepsin B and the 'endogenous thiol proteinase' are different enzymes produced by distinct genes (see below). However, Olstein & Liener [31] purified the two enzymes from liver and sarcoma 24- and 6-fold respectively, that is, considerably less than the cathepsin B purified for the present studies. Our results conform with those reporting only one gene for cathepsin B [32], as well as with observations indicating that there are no significant differences between normal cathepsin L [33] and H [34] and their tumour counterparts.

Mature human cathepsin B has been reported to exist in two forms: a single-chain form of ~ 30 kDa and a double-chain form with a heavy chain of ~ 25 kDa and a light chain of ~ 5 kDa [4]. We detected both single- and double-chain forms in fractions from both normal human liver and human tumours that contain cathepsin B activity (Fig. 1). In addition, we detected two isoforms of the heavy chain of the double-chain form of cathepsin B (Fig. 1). We confirmed that all three forms were cathepsin B by reactivity with monospecific antibodies (Figs. 2 and 3) and by N-terminal sequencing (Fig. 4). Cathepsin B purified from normal human kidney also exists as three forms of similar molecular mass [35].

In the present study the major peak of cathepsin B activity was associated with fractions containing only the double-chain form of the enzyme (Fig. 1). This contrasts with the report by Hanewinkel *et al.* [9] that the single-chain form of human fibroblast cathepsin B is more active than the double-chain form. However, purified proteins were not assayed in the latter study. In a recent study in which a comprehensive kinetic analysis was carried out with the single-chain form of recombinant rat cathepsin B, the kinetic constants are similar to those reported here for double-chain forms of human cathepsin B [36].

Takahashi *et al.* [37] have contended that cathepsin B is an exopeptidase without endopeptidase activity, on the basis of its inability to cleave certain peptide and protein substrates (with the exception of those for dipeptidyl dipeptidases and aminopeptidases). A recent report by Mason [38] on the reaction of cathepsin B with α_2 -macroglobulin and results from our laboratory on the degradation of laminin, collagen type IV and fibronectin by cathepsin B [39,40] have re-established that cathepsin B has endopeptidase activity.

Cathepsin B has been reported to have low affinity towards Con A [4,30], a finding consistent with the reports that cathepsin B has abbreviated forms of the high-mannose type oligosaccharides normally found on lysosomal enzymes [29,30,41]. The trimming of the high-mannose oligosaccharide chains on cathepsin B may occur during proteolytic processing to the mature form, since Nishimura *et al.* [42] have separated rat liver procathepsin B from mature cathepsin B by exploiting the ability of procathepsin B to bind to Con A. However, we have demonstrated that the difference between the 25 kDa and 26 kDa heavy chains of human liver and tumour cathepsin B was an ~ 1 kDa N-linked high-mannose-type oligosaccharide moiety. Khalfan [43] contends that active cathepsin B has no affinity for Con A, although the data he presents indicate that ~ 50% of cathepsin B binds to Con A-Sepharose. A fraction of the purified mature double-chain cathepsin B from human sarcoma, corresponding to the 26 kDa band on SDS/PAGE and to the fractions with highest activity, bound to a Con A-Sepharose

column (Fig. 6). Thus a portion of the mature cathepsin B has the high-mannose carbohydrates necessary for binding to Con A.

Cathepsin B, normally present in the lysosomes, is believed to have optimal activity under the acidic conditions found in the lysosomal lumen [4]. Cathepsin B from normal liver has been reported to have optimal activity at about pH 5.5–6.5, but to be inactive and unstable at pH values at or above 7.0 [4,21,44–46]. In the present study, cathepsin B from both normal liver and tumour tissues had considerable activity at about neutral pH. The kinetic data (Table 2) indicated that cathepsin B from both liver and tumour tissues had greater activity when assayed at neutral pH than acidic pH (consistent with results obtained from recombinant rat cathepsin B [36]). Nevertheless, the kinetic data were obtained at 24 °C against synthetic substrates, conditions not encountered *in vivo*. Previous work by Willenbrock & Brocklehurst [46] and Maciewicz *et al.* [47] have shown that activity and stability of cathepsin B at higher pH are temperature- and substrate-dependent. Willenbrock & Brocklehurst [46] demonstrated that cathepsin B is unstable at 37 °C when tested against synthetic substrates. Maciewicz *et al.* [47], on the other hand, found that cathepsin B is active at neutral pH at 37 °C when tested against the protein substrates collagens types II and XI. We have also shown cathepsin B from both normal and tumour tissues to be active against the protein substrates laminin and fibronectin at neutral pH at 37 °C [40]. Under physiological conditions of pH and temperature, cathepsin B also autodegrades at a higher rate than at acidic pH, resulting in a loss of activity [40]. This autodegradation and therefore the loss of activity are slowed in the presence of alternative protein substrates [40]. Thus, although pH and temperature may cause conformational changes in cathepsin B *in vitro* which can render the enzyme unstable, the larger protein substrates present *in vivo* may stabilize the enzyme so that it retains activity at neutral pH.

We thank Dr. John Crissman (Department of Pathology, Wayne State University, Detroit, MI, U.S.A.), Dr. Vito Turk (Department of Biochemistry, Josef Stefan Institute, Ljubljana, Slovenia) and Dr. Daniel H. Rich (School of Pharmacy, University of Wisconsin, Madison, WI, U.S.A.) for providing human tissues (liver and tumours), cathepsin L and synthetic peptide inhibitors respectively. This study was supported by U.S. Public Health Service Grant CA 36481.

REFERENCES

1. Sloane, B. F. (1990) *Semin, Cancer Biol.* **1**, 137–152
2. Sloane, B. F., Moin, K., Krepela, E. & Rozhin, J. (1990) *Cancer Metastasis Rev.* **9**, 333–352
3. Sloane, B. F., Moin, K. & Lah, T. T. (1992) in *Aspects of the Biochemistry and Molecular Biology of Tumors* (Pretlow, T. G., & Pretlow, T. P., eds.), Academic Press, New York, in the press
4. Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561
5. Ritonja, A., Popovic, T., Turk, V., Wiedenmann, K. & Macheidt, W. (1985) *FEBS Lett.* **181**, 169–172
6. Musil, D., Zucic, D., Turk, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katanuma, N. & Bode, W. (1991) *EMBO J.* **10**, 2321–2330
7. Sly, W. S. & Fischer, H. D. (1982) *J. Cell. Biochem.* **18**, 67–85
8. Kornfeld, S. (1987) *FASEB J.* **1**, 462–468
9. Hanewinkel, H., Glossl, J. & Kresse, H. (1987) *J. Biol. Chem.* **262**, 12351–12355
10. Reitman, M. L., Varki, A. & Kornfeld, S. (1981) *J. Clin. Invest.* **67**, 1574–1579
11. Neufeld, E. F. (1981) in *Lysosomes and Lysosomal Storage Diseases* (Callahan, J. W. & Lowden, J. A., eds.), pp. 115–129, Raven Press, New York
12. Van Dongen, J. M., Willensen, R., Ginns, E. I., Sips, H. J., Tager, J. M., Barranger, J. A. & Reuser, A. J. (1985) *Eur. J. Cell Biol.* **39**, 179–189
13. Recklies, A. D., Tiltman, K. J., Stoker, T. A. M. & Poole, A. R. (1980) *Cancer Res.* **40**, 550–556

14. Sloane, B. F., Honn, K. V., Sadler, J. G., Turner, W. A., Kimpson, J. J. & Taylor, J. D. (1981) *Cancer Res.* **42**, 980–986
15. Mort, J. S., Leduc, M. & Recklies, A. D. (1981) *Biochim. Biophys. Acta* **662**, 173–180
16. Recklies, A. D., White, C., Mitchell, J. & Poole, A. R. (1985) *Cancer Res.* **45**, 2294–2301
17. Krepela, E., Bartek, J., Skalkova, D., Vicar, J., Rasnick, D., Taylor-Papadimitriou, J. & Hallows, R. C. (1987) *J. Cell Sci.* **87**, 145–154
18. Erdel, M., Trefz, G., Spiess, E., Habermaas, S., Spring, H., Lah, T. & Ebert, W. (1990) *J. Histochem. Cytochem.* **38**, 1313–1321
19. Sloane, B. F., Rozhin, J., Krepela, E., Ziegler, G. & Sameni, M. (1991) *Biomed. Biochim. Acta* **50**, 549–554
20. Sloane, B. F., Rozhin, J., Hatfield, J. S., Crissman, J. D. & Honn, K. V. (1987) *Exp. Cell Biol.* **55**, 209–224
21. Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
22. Rich, D. H., Brown, M. A. & Barrett, A. J. (1986) *Biochem. J.* **235**, 731–734
23. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
24. Merril, C. R., Goldman, D. & Van Keuren, M. L. (1984) *Methods Enzymol.* **104**, 441–447
25. Hunkapillar, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236
26. Tarentino, A. L., Gomez, C. M. & Plummer, T. H. (1985) *Biochemistry* **24**, 4665–4671
27. Nuck, R., Zimmermann, M., Sauvageot, D., Josic, D. & Reutter, W. (1990) *Glycoconjugate J.* **7**, 279–286
28. Nicklin, M. J. H. & Barrett, A. J. (1984) *Biochem. J.* **223**, 245–253
29. Takahashi, T., Schmidt, P. G. & Tang, J. (1984) *J. Biol. Chem.* **259**, 6059–6062
30. Taniguchi, T., Mizuochi, T., Towatari, T., Katunuma, N. & Kobata, A. (1985) *J. Biochem. (Tokyo)* **97**, 973–976
31. Olstein, A. D. & Liener, I. E. (1983) *J. Biol. Chem.* **258**, 11049–11056
32. Fong, D., Calhoun, D. H., Hsieh, W. T., Lee, B. & Wells, R. D. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1909–1913
33. Stearns, N. A., Dong, J., Pan, J., Brenner, D. A. & Sahagian, G. G. (1990) *Arch. Biochem. Biophys.* **283**, 447–457
34. Tsushima, H., Ueki, A., Matsuoka, Y., Mihara, H. & Hopsu-Havu, V. K. (1991) *Int. J. Cancer* **48**, 726–732
35. Wang, P. H., Do, Y. S., Macaulay, L., Shinagawa, T., Anderson, P. W., Baxter, J. D. & Hsueh, W. (1991) *J. Biol. Chem.* **266**, 12633–12638
36. Hasnain, S., Hiram, T., Tam, A. & Mort, J. S. (1992) *J. Biol. Chem.* **267**, 4713–4721
37. Takahashi, T., Dehdarani, A. H., Yonezawa, S. & Tang, J. (1986) *J. Biol. Chem.* **261**, 9375–9381
38. Mason, R. W. (1989) *Arch. Biochem. Biophys.* **273**, 367–374
39. Lah, T. T., Buck, M. R., Honn, K. V., Crissman, J. D., Rao, N. C., Liotta, L. A. & Sloane, B. F. (1989) *Clin. Exp. Metastasis* **7**, 461–468
40. Buck, M. R., Karustis, D. G., Day, N. A., Honn, K. V. & Sloane, B. F. (1992) *Biochem. J.* **282**, 273–278
41. Takahashi, T., Yonezawa, S., Dehdarani, A. H. & Tang, J. (1986) *J. Biol. Chem.* **261**, 9368–9374
42. Nishimura, Y., Kawabata, T. & Kato, K. (1988) *Arch. Biochem. Biophys.* **261**, 64–71
43. Khalfan, H. A. (1991) *Cell Biochem. Function* **9**, 55–62
44. Towatari, T., Kawabata, Y. & Katunuma, N. (1979) *Eur. J. Biochem.* **102**, 279–289
45. Takahashi, S., Murakami, K. & Miyake, Y. (1981) *J. Biochem. (Tokyo)* **90**, 1677–1684
46. Willenbrock, F. & Brocklehurst, K. (1985) *Biochem. J.* **227**, 511–519
47. Maciewicz, R. A., Wotton, S. F., Etherington, D. J. & Duance, V. C. (1990) *FEBS Lett.* **269**, 189–193

Received 11 November 1991/7 January 1992; accepted 15 January 1992