FOR THE RECORD

Characterization of recombinant human cathepsin B expressed at high levels in baculovirus

PAUL M. STEED, DANIEL LASALA, JEFFREY LIEBMAN, ANDREW WIGG,
KIRK CLARK, AND ANIA K. KNAP

Research Department, Novartis Pharmaceuticals, 556 Morris Avenue, Summit, New Jersey 07901

(RECEIVED April 22, 1998; ACCEPTED May 19, 1998)

Abstract: The lysosomal cysteine protease cathepsin B has been studied intensely for many years because of its unique characteristics and its potential involvement in disease states. A reproducible, high yield expression system for active recombinant protein is key to biochemical and biophysical studies as well as rational drug design. Although several microbial and mammalian expression systems for recombinant human cathepsin B have been described, these have been limited by low or variable yields. Further, in some of these systems hyper-glycosylation of the enzyme near the active site affects its activity. We describe a baculovirus expression system and purification scheme that solve all of these problems. Yields of active, protected enzyme were reproducibly in excess of 25 mg/L. Since this protein was not hyper-glycosylated, it had greater activity than cathepsin B produced in yeast systems as indicated by a threefold increase in K_{cat} . In addition, the biophysical properties of the baculovirus-expressed cathepsin B, as measured by dynamic light scattering, were more amenable to crystallographic study since the data indicated proteins of more uniform size. Therefore, this system for the production of recombinant human cathepsin B constitutes a major improvement in both quantity and quality over those previously reported. Further, we demonstrate that the manner of expression and purification of this enzyme has profound effects on its kinetic and physical parameters.

Keywords: cathepsin B; cysteine protease; expression; kinetics; purification; structure

Cathepsin B (EC 3.4.22.1) is a lysosomal cysteine protease involved in cellular protein turnover (Kirschke & Barrett, 1987). Among the members of this class of proteases, cathepsin B has been extensively studied not only because of its high abundance, but also because it has unique features, exhibiting both endopeptidase and peptidyl dipeptidase activity (Aronson & Barrett, 1978; Fosang et al., 1992). Further, this enzyme has been implicated in

several pathological conditions including arthritis and tumor metastasis. Levels of cathepsin B are increased in human osteoarthritic cartilage (Martel-Pelletier et al., 1990), and known inhibitors of cathepsin B have been reported to ameliorate experimentally induced arthritis (Esser et al., 1993). Elevated levels of cathepsin B have also been implicated in tumor progression (Sloane, 1990). These and other investigations have kindled interest in determining the molecular structure of this cysteine protease at high resolution for use in mechanistic studies as well as in rational drug design. Such studies have begun to delineate the contribution of defined domains to enzyme activity (Fox et al., 1992; Lily et al., 1997). Notably, crystallographic analyses of recombinant cathepsin B and other members of the papain family have revealed the presence of an additional structural feature termed the "occluding loop," and mutation analysis has shown this structure to be involved in the exopeptidase and endopeptidase activities of the enzyme (Lily et al., 1997; Nagler et al., 1997).

To provide sufficient enzyme for biophysical studies, several expression systems have been developed. Production of recombinant human cathepsin B from *Escherichia coli* has been reported at a yield of 3 mg/L culture, but refolding and reoxidation was required to obtain active enzyme (Kuhelj et al., 1995). Mammalian cells have been reported to yield recombinant human procathepsin B at ~1 mg purified enzyme per 5×10^8 cells (Ren et al., 1996). High yields of recombinant rat cathepsin B have been reported in *Saccharomyces cerevisiae* (Hasnain et al., 1992) and *Pichia pastoris* (Sivaraman et al., 1996) but attempts to produce large quantities of recombinant human cathepsin B in these systems were not successful (Hasnain et al., 1992; Lily et al., 1997). In our hands, despite extensive efforts to optimize the expression of human cathepsin B in *P. pastoris*, yields have remained low and variable. Because several other lysosomal cysteine proteases, including cathepsin S and cathepsin K, have been successfully expressed in baculovirus (Bossard et al., 1996; Bromme & McGrath, 1996), we examined this approach to the expression of human cathepsin B. An additional factor affecting yield from expression systems for thiol proteases is the stability of the enzyme during storage. Specifically, the active site cysteine of the mature protease is easily oxidized causing enzyme inactivation (Hasnain et al., 1992). To

Reprint requests to: Paul M. Steed, Research Department, Novartis Pharmaceuticals, 556 Morris Avenue, Summit, New Jersey 07901; e-mail: paul.steed@pharma.novartis.com.

Table 1. Purification of recombinant human cathepsin B from sample *P. pastoris* and Sf9-baculovirus cultures

Sample	Sf9-baculovirus media			<i>P. pastoris</i> media		
	Total protein (mg)	Total activity (Units) ^a	Units/mg	Total protein (mg)	Total activity (Units) ^a	Units/mg
Media	560	9,884	18	nd ^b	nd	nd
Activated	206	6,791	32	99	39 ^c	0.4
Endo EF treatment	nd	nd	nd	nd	37	nd
SP-Sepharose	118	7,730	66	nd	559	nd
Affinity	27	8,005	293	4.7	248	53

^aUnits are expressed as $\mu\text{M}/\text{min}$.^bOf 16 batches, this was the highest yield and >50% had barely detectable levels of activity.^cnd = not determined.

avoid this problem, an affinity resin can be employed which, when the protein is eluted, allows for storage with the active site blocked (Rich et al., 1986).

Here, we describe a baculovirus expression system and an accompanying rapid purification scheme that provides recombinant human cathepsin B in high yields suitable for biochemical and crystallographic studies. Further, we determined that the level of glycosylation of recombinant human cathepsin B depends on the preparation scheme utilized and has a profound effect on the activity and physical properties of the recombinant enzyme. This observation has profound implications regarding the biochemical and biophysical analyses of recombinant cathepsin B.

Results and discussion: *Expression and purification of recombinant human cathepsin B:* A cDNA clone encoding human cathepsin B was obtained via rtPCR and used to construct systems utilizing the methylotrophic yeast *P. pastoris* for the expression of recombinant human cathepsin B. Unfortunately, in our hands this system was inadequate because the yields were low and extremely variable. Numerous conditions and media were tested (with the assistance of the fermentation group at the Waksman Institute for Microbiology, Rutgers University) for both standard and fermenter cultures. The addition of agents that reversibly block the active site cysteine did not improve yields and the use of media buffered to higher pH (to prevent autoprocessing of procathesin B) dramatically reduced yields. The results for *P. pastoris* expression shown in Table 1 were obtained from the best of 16 batches, using the optimal conditions, half of which failed to show detectable enzyme activity.

As an alternative to yeast expression, baculovirus expression vectors were constructed using the cDNA clone encoding human cathepsin B. Yields of activated product from the Sf9-baculovirus expression system were consistently greater by two orders of magnitude (Table 2). Enzyme activity in Sf9-baculovirus medium was more than 20-fold greater than that from the corresponding cell pellets, as would be expected since there is a signal peptide in the sequence of pre-procathepsin B (data not shown). During optimization of yields from the Sf9-baculovirus expression system, it was found that the presence of fetal bovine serum suppressed the production of cathepsin B (Table 2), that supplementation of serum-free medium with added 0.1% pluronic acid increased yields two-fold (Table 2), and that optimal multiplicities of infection were as low as 0.003. We have yet to determine the reason for this low

MOI optima; however, similar results are seen with the expression of rhCathepsin S in this system and this phenomenon does not appear to be specific to thiol proteases (J. Liebman et al., unpubl. obs.).

For the purification of recombinant human cathepsin B, we found that S-Sepharose fast flow resin was the most efficient initial step because it was rapid, had adequate resolution, and the majority of material in the culture media, especially from *Pichia*, was not retained by the column. For purification of the protease to near homogeneity, we employed the affinity resin Sepharose-Phe-Gly-Sc; however, we found that the addition of DTT to the enzyme prior to loading on the column as described (Rich et al., 1986) caused a loss in yield of up to 80%. To avoid this problem, we added enzyme and DTT to the resin simultaneously. When purified from *P. pastoris* or Sf9-baculovirus media, procathesin B migrated in SDS PAGE at a position consistent with ~36 kDa while activated cathepsin B migrated as a single band of ~29.5 kDa (Fig. 1). The inferred size of this protein species corresponds to that expected from the cDNA sequence and previous reports (Iily et al., 1997). The baculovirus-expressed cathepsin B was purified to near homogeneity in two steps, as evidenced by SDS-PAGE analysis (Fig. 1). Similar analysis of the *Pichia*-derived enzyme revealed the probable source of the variable yield found with this system. While most or all of the cathepsin B obtained from the baculovirus culture is in the pro form (>90%, Fig. 1), it is predominantly in mature form in the *Pichia* cultures (not shown) indicating autoactivation of the enzyme. This phenomenon may be due to the significantly lower pH required for the *Pichia* expression or there may be an endogenous inhibitor in the baculovirus

Table 2. Comparison of expression methods for recombinant human cathepsin B

Expression method	Product total activity (mU/mL)	Product activated total activity (mU/mL) ^a
<i>P. pastoris</i>	21.2	24.3
Baculovirus/ 10% FBS	5	6.3
Baculovirus/serum free	116	3,889
Baculovirus/serum free/pluronic acid	167	7,991

^aUnits are expressed as $\mu\text{M}/\text{min}$.

Fig. 1. Purification of recombinant human cathepsin B expressed in baculovirus. Expression conditions, enzyme purification, SDS-PAGE, and visualization are described in Materials and methods. Sizes corresponding to pro and mature cathepsin B are indicated by arrows. Lane 1, crude expression media; lane 2, crude expression media treated to activate cathepsin B; lane 3, eluent from cation exchange chromatography; lane 4, eluent from affinity chromatography; lane 5, purified recombinant human cathepsin B expressed in *P. pastoris*.

system. Since autoactivation likely affects yield by autocatalysis and/or oxidation of the product and the acidic conditions required for optimal *Pichia* expression probably contributes to the autoactivation. Our data indicate that expression of recombinant human cathepsin B in baculovirus eliminates this problem. Oxidation of the active site cysteine is also a potential problem for long term storage of the enzyme. An additional advantage of our methods is that the elution of cathepsin B from the affinity column with 2,2'-dithiodipyridine blocks and protects the active site cysteine. As a result, our enzyme is stable at -80°C for at least 6 months.

Kinetic and physical characterization of recombinant human cathepsin B: Kinetic analyses of purified cathepsin B from *P. pastoris* and from the Sf9-baculovirus expression system indicated that the level of glycosylation of the enzyme profoundly affected the activity. There was no significant difference in K_m between the enzymes produced in *Pichia* (with or without endoglycosidase

treatment), baculovirus, and *S. cerevisiae* (with site removed; Table 3). However, cathepsin B lacking sugar residues had a two-fold to threefold higher K_{cat} . This finding is consistent with the observation that alterations in the occluding loop of cathepsin B affect both endo and exopeptidase activity (Lily et al., 1997; Nagler et al., 1997) because the single glycosylation site on the enzyme is located on the occluding loop (Hasnain et al., 1992). Further, alterations of the sequence that eliminate this site, as with the *S. cerevisiae*-expressed cathepsin B (Table 3), also result in a lower K_{cat} .

To evaluate the recombinant human cathepsin B preparations for their suitability for biophysical studies, they were analyzed by dynamic light scattering (Table 3). In these experiments, the baseline parameter measures how well the data fit a curve for a monodispersed size distribution while the sum of squares error represents the error associated with this function. The data obtained from the *Pichia*-expressed cathepsin B were best represented by a monodisperse distribution with a baseline slightly higher than expected for a true monodisperse sample (i.e., baseline <1.001). In addition, the diffusion coefficient was relatively small and corresponded to a species larger than the 29 kDa size expected for the mature recombinant human cathepsin B protein. When the *Pichia*-expressed recombinant human cathepsin B was treated with the endoglycosidases, Endo-H and Endo-F during purification, the light scattering results were markedly different. The data were clearly monodisperse with a narrow size distribution (Table 3). The estimated polydispersity of 0.2 nm was less than 10% of the hydrodynamic radius, 2.4 nm. The cathepsin B derived from the Sf9-baculovirus expression system yielded similar results without endoglycosidase treatment.

In conclusion, we have found that while expression in a yeast system can provide sufficient quantity and quality of recombinant human cathepsin B for enzymatic and biophysical studies, the yields are highly variable and the hyper-glycosylation of the enzyme alters its kinetic and physical properties. Since the single N-linked glycosylation site resides on the occluding loop proximal to the active site of the enzyme, alterations in the kinetic properties as a result of hyper-glycosylation were not surprising. These sugar residues can be removed enzymatically or by mutation of the coding region, though such mutations also alter the kinetic properties of the enzyme (data not shown). Therefore, it is clear that the manner of production and preparation of recombinant cathepsin B has profound effects on enzyme activity. This phenomenon also

Table 3. Kinetic and biophysical parameters of recombinant human cathepsin B

Expression method	Dynamic light scattering							
	Kinetics		Diffusion coefficient (m ² /s)	Hydrodynamic radius (nm)	Polydispersity (nm)	Estimated molecular weight (kDa)	Baseline	Sum of squares error
	K_M (mM)	K_{cat} (s ⁻¹)						
<i>P. pastoris</i>	563 ± 35	16 ± 0.3	648 × 10 ⁻¹³	3.3	0.6	54	1.002	1.37
<i>P. pastoris</i> /endo H/F treated	393 ± 58	35 ± 2	905 × 10 ⁻¹³	2.4	0.2	26	1.001	1.22
Baculovirus	467 ± 18	43 ± 19	nd	nd	nd	nd	nd	nd
<i>S. cerevisiae</i> /(linkage mutated as in Hasnain et al., 1992) ^a	357 ± 55	5.8 ± 0.3	905 × 10 ⁻¹³	2.5	0.3	26	1.001	3.18

^aEnzyme kindly provided by J. Mort.

extends to the biophysical properties of the enzyme. Our data show that the baculovirus-prepared cathepsin B exhibits low polydispersity, indicating a greater likelihood of successful crystallization, which we have directly confirmed (data not shown). Expression of recombinant human cathepsin B in baculovirus avoids the problems of variability and glycosylation while providing dramatically higher yields. The ability to purify to homogeneity large quantities of stable highly active recombinant human cathepsin B makes this expression/purification system ideally suited for production of protein for both biochemical and biophysical studies.

Materials and methods: *Cloning of human preprocathepsin B and construction of expression vectors:* Complementary DNA (cDNA) encoding human pre-procathepsin B was amplified from human liver cDNA produced by RT-PCR (Clontech, Palo Alto, California). The amplification primers corresponded to the first 20 and last 21 bases of the coding region of human preprocathepsin, respectively, according to the sequence HUMCATHB (GenBank accession no. L16510), and contained additional sequences at the ends that facilitated subsequent molecular cloning. Sequencing of the cloned PCR products was accomplished using AmpliTaq DNA polymerase, FS (Perkin Elmer Corp., Foster City, California) for sequencing reactions and an ABI 377 automated sequencer (Perkin Elmer) for electrophoresis and data collection.

For expression in yeast, the cDNA was subcloned into the expression vector pPIC9 (Invitrogen, Portland, Oregon) which introduces the *S. cerevisiae* alpha mating factor signal sequence at the 5' end of the cDNA. The constructs were recombined onto the chromosomes of the methylotrophic yeast *P. pastoris* by allele replacement with the AOX1 locus according to the vendor's recommendation. Baculovirus expression constructs were prepared using the BAC-to-BAC baculovirus expression system according to the vendor's instructions (Life Technologies, Gaithersburg, Maryland). Briefly, *E. coli* DH10Bac cells (containing a modified baculovirus, or bacmid) were co-transformed with the donor plasmid containing the preprocathepsin B cDNA. Recombinant bacmids, formed via transposition, were isolated using a β -gal screen. The bacmid was harvested and used for infection of insect cells and subsequent propagation of viral stocks.

Assay for cathepsin B activity: In a 500 μ L quartz cuvette at 25°C, 10 μ L of enzyme sample was combined with 480 μ L of buffer (88 mM KH_2PO_4 , 12 mM Na_2HPO_4 , 1.33 mM EDTA, 2.7 mM DTT, 0.03% Brij 35, pH 5.8) and 10 μ L of the substrate stock solution of Z-Arg-Arg-AMC (Peptides International, Louisville, Kentucky; 80 mM in DMSO). Excitation at 380 nm and emission at 440 nm were monitored on a LS50B Luminescence Spectrometer (Perkin Elmer) for activity determinations. For activation of baculovirus-derived samples prior to purification, these samples were applied to PD-10 columns (Pharmacia, Piscataway, New Jersey) pre-equilibrated with 50 mM sodium acetate, pH 5.0 with 1 mM EDTA, and were eluted in 3.5 mL of the same buffer. Samples were stored overnight at 4°C to allow full activation in this buffer. This assay was used to monitor purification of the enzyme and to study the kinetic characteristics of the recombinant products. Cathepsin B was activated by dialysis to SP-Sepharose Buffer (pH 5.0) and incubation at 4°C overnight. For kinetic analyses, the amount of active enzyme was determined by titration with the thiol protease inhibitor E64 as previously described (Kirschke & Barrett, 1987).

Expression of recombinant human cathepsin B in yeast: Materials and sources were: yeast nitrogen base without amino acids, yeast extract, agar, and dextrose (Difco, Detroit, Michigan); glycerol (EM Science, Gibbstown, New Jersey); biotin, methyl- α -D-glucopyranoside, and sodium acetate (Sigma, St. Louis, Missouri); peptone (Life Technologies, Gaithersburg, Maryland). The recombinant human cathepsin B expression strains, stored at -80°C , were streaked out on minimal dextrose medium plates (1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 1.0% dextrose agar plates) and incubated at 30°C to obtain isolated colonies. A single colony was used to inoculate 50 mL of buffered glycerol-complex medium (BMGY: 1.0% yeast extract, 2.0% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 1.0% glycerol; pH 6.0), which was incubated overnight with shaking and used to inoculate 10 L of BMGY. After two days of incubation at 30°C with shaking, the cultures were centrifuged at $1,500 \times g$ for 10 min. Cells were resuspended in 5 L of buffered methanol-complex medium, pH 4.5 (BMMY: identical to BMGY except that 0.5% methanol was substituted for glycerol) and incubated at 30°C with continuous shaking for five days. Cathepsin B expression was induced on days 1, 2, and 4 of incubation by adding 20 mL methanol per 4 L. At the end of day 5, the cultures were centrifuged at $4,500 \times g$, and the supernatant was concentrated approximately 10-fold using an 8,000 MW cutoff cartridge in a Filtron Centrimate concentrator (Northborough, Massachusetts). The concentrated medium was then dialyzed against 100 $\times$ excess of SP-Sepharose buffer (50 mM sodium acetate pH 5.0, 1.0 mM EDTA).

Expression of recombinant human cathepsin B in baculovirus: Amplified cathepsin B baculoviral stock titers were estimated from plaque assays (O'Reilly et al., 1992). Insect cells (Sf9 strain; Pharmingen, San Diego, California) were cultured in Grace's medium containing 10% fetal bovine serum, 0.1% pluronic acid, antibiotics and amphotocerin (Life Technologies, Gaithersburg, Maryland) and grown at 125–300 mL culture volume in shaker flasks at 27°C. After cells had reached a density of $\sim 1.5\text{--}2.5 \times 10^6$ cells/mL, they were resuspended in fresh medium at a final density of 1×10^6 cells/mL and cathepsin B baculoviral stock was added as indicated below. Cultures were incubated at 27°C for 96–120 h. In some cases, medium was changed to Sf-900II serum-free medium (Life Technologies) 24 h after inoculation. At harvest, samples were clarified and cell pellets and medium were stored at -70°C for subsequent purification and assay.

Purification of recombinant human cathepsin B by cation exchange chromatography: The recombinant human cathepsin B, exchanged to SP-Sepharose Buffer, was loaded at a flow rate of 5 mL/min onto a 20×300 mm Waters (Milford, Massachusetts) AP-2 column packed with ~ 90 mL of SP-Sepharose fast flow resin (Pharmacia, Piscataway, New Jersey) and equilibrated with SP-Sepharose Buffer at 4°C. The column was washed with two column volumes of SP-Sepharose Buffer, and cathepsin B activity was eluted with a linear gradient of two column volumes of SP-Sepharose Buffer from 0 to 1.0 M NaCl.

Removal of sugars from Pichia-expressed recombinant human cathepsin B: Though there was no detectable glycosylation of recombinant human cathepsin B in the baculovirus expression system,

as determined by SDS-PAGE and mass spectroscopy analysis (not shown), the enzyme expressed in *Pichia* was found to be extensively glycosylated on the single N-linked glycosylation site. For some experiments, most of the sugar residues were removed from purified enzyme as follows. The *Pichia*-expressed recombinant human cathepsin B activity from the cation exchange column was concentrated approximately 10-fold in an Amicon stirred cell concentrator (Amicon, Beverly, Massachusetts) using a YM10 membrane. To this sample, 200 mUnits of Endoglycosidase H (Boehringer Mannheim, Indianapolis, Indiana) was added; the material was incubated at 37°C for 3 h with gentle agitation and dialyzed overnight at 4°C against 50 mM sodium phosphate pH 7.2. At a flow rate of 2.0 mL/min, this material was passed through a 10 × 200 mm Waters AP-1 column packed with ConA-Sepharose (Pharmacia) previously equilibrated with ConA buffer (50 mM sodium phosphate pH 6.0, 0.5 M NaCl). The cathepsin B activity flowing through the column was pooled and EDTA added to a final concentration of 1.0 mM.

Affinity purification of recombinant human cathepsin B: All chemicals were obtained from Sigma. Sepharose-Phe-Gly-Sc resin was prepared as previously described (Rich et al., 1986) and packed into a 50 × 200 mm Waters AP-1 column. The column was equilibrated with Affinity A buffer (50 mM sodium phosphate pH 6.0, 1.0 mM EDTA) at a flow rate of 10 mL/min at 4°C. The cathepsin B activity was loaded onto the column concomitantly with DTT to a final concentration of 2.0 mM. Affinity A buffer with 2.0 mM DTT was then loaded and the column was washed with four column volumes of Affinity A buffer, four column volumes of Affinity A buffer with 0.5 M NaCl, and four column volumes of 50 mM sodium formate pH 4.0. To elute the cathepsin B, Affinity B buffer (50 mM sodium formate pH 4.0, 1.5 mM 2,2'-dithiodipyridine) was loaded onto the column, left overnight and eluted with this buffer the following day. The overnight elution ensures that all of the material is removed from the resin. The two-step purification protocol, SP-Sepharose, and affinity purification on Sepharose-Phe-Gly-Sc, resulted in a greater than 10-fold purification (Table 2) of baculovirus-expressed recombinant human cathepsin B to homogeneity, as determined by SDS PAGE electrophoresis.

SDS PAGE electrophoresis and protein visualization: Aliquots of active fractions from the purification steps were concentrated in Centricon 10 concentrators [Amicon, W.R. Grace & protein gel stain (Molecular Co., Danvers, Massachusetts) and run on pre-cast 12% SDS PAGE gels (Bio-Rad); gels were stained with SYPRO Orange Probes, Eugene, Oregon], and visualized on a Molecular Dynamics Storm fluorescence scanning system.

Dynamic light scattering: The purified recombinant cathepsin B derived from *P. pastoris* and baculovirus was analyzed by dynamic

light scattering. The translational diffusion coefficient was measured using a DynaPro-801 detector (Protein Solutions, Inc. Charlottesville, Virginia). Data analysis was performed with the AutoPro software as supplied with the DynaPro-801. Protein samples were concentrated to 2 mg/mL (sodium acetate buffer, 250 mM KCl, pH 5.7) and were injected into the detector through a 0.02 µm Anotop filter. The data were collected at 296 K, and at least 20 measurements were averaged for the final summary statistics. The hydrodynamic radius was estimated using the Stokes-Einstein equation $R_H = kT/6\pi\eta D_T$ where k is Boltzmann's constant, and the viscosity, η , was estimated to be 1.019 centipoise. The molecular weight was estimated from an empirical relationship between R_H and molecular weight using a number of globular proteins as standards.

References

- Aronson NN, Barrett AJ. 1978. The specificity of cathepsin B: Hydrolysis of glucagon at the C-terminus by a peptidyl dipeptidase mechanism. *Biochem J* 171:759-765.
- Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY, Hanning CR, Jones C, Kurdyla JT, McNulty DE, Drake FH, Gowen M, Levy MA. 1996. Proteolytic activity of human osteoclast cathepsin K. *J Biol Chem* 271:12517-12524.
- Bromme D, McGrath ME. 1996. High level expression and crystallization of recombinant human cathepsin S. *Protein Sci* 5:789-791.
- Esser RE, Watts LM, Angelo RA, Thornburg LP, Prior JJ, Palmer JT. 1993. The effects of flouromethyl ketone inhibitors of cathepsin B on adjuvant induced arthritis. *J Rheum* 20:1176-1180.
- Fosang A, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA. 1992. The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases and cathepsin B. *J Biol Chem* 267:19470-19474.
- Fox T, Miguel ED, Storer AC. 1992. Potent slow-binding inhibition of cathepsin B by its propeptide. *Biochemistry* 31:12571-12576.
- Hasnain S, Hiram T, Tam A, Mort JS. 1992. Characterization of recombinant rat cathepsin B and nonglycosylated mutants expressed in yeast. *J Biol Chem* 267:4713-4721.
- Ily C, Quraishi P, Wang J, Purisima E, Vernet T, Mort JS. 1997. Role of the occluding loop in cathepsin B activity. *J Biol Chem* 272:1197-1202.
- Kirschke H, Barrett AJ. 1987. Lysosomal cysteine proteases. In: Glaumann H, Ballard FJ, eds. *Lysosomes: Their role in protein breakdown*. London: Academic Press. pp 193-238.
- Kuhelj R, Dolinar M, Pungercar J, Turk V. 1995. The preparation of catalytically active human cathepsin B from its precursor expressed in *Escherichia coli* in the form of inclusion bodies. *Eur J Biochem* 229:533-539.
- Martel-Pelletier J, Cloutier JM, Pelletier JP. 1990. Cathepsin B and cysteine protease inhibitors in human osteoarthritis. *J Orthop Res* 8:336-344.
- Nagler DK, Storer AC, Bortaro FCV, Carmona E, Juliano L, Menard R. 1997. Major increase in endopeptidase activity of human cathepsin B upon removal of occluding loop contacts. *Biochemistry* 36:12608-12615.
- O'Reilly DR, Miller LK, Luckow VA. 1992. *Baculovirus expression: A laboratory manual*. New York: W.H. Freeman.
- Ren WP, Fridman R, Qabrecky JR, Morris LD, Day NA, Sloane BF. 1996. Expression of functional recombinant human procathepsin B in mammalian cells. *Biochem J* 319:793-800.
- Rich DH, Brown MA, Barrett AJ. 1986. Purification of cathepsin B by a new form of affinity chromatography. *Biochem J* 235:731-734.
- Sivaraman J, Colombe R, Magny MC, Mason P, Mort JS, Cygler M. 1996. Crystallization of rat procathepsin B. *Acta Cryst D52*:874-875.
- Sloane BF. 1990. Cathepsin B and cystatins: Evidence for a role in cancer progression. *Semin Cancer Biol* 1:137-152.