Cathepsin B of Schistosoma mansoni

PURIFICATION AND ACTIVATION OF THE RECOMBINANT PROENZYME SECRETED BY SACCHAROMYCES CEREVISIAE*

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Procathepsin B from the parasitic trematode Schistosoma mansoni was expressed as a glycosylation-minus mutant in yeast cells and purified by means of a histidine affinity tag which was added to the carboxyl terminus of the recombinant protein. The purified zymogen underwent autoprocessing but required an assisting protease for activation. Pepsin-activated schistosomal cathepsin B was further characterized with the cathepsin B-specific substrates (Z)-Arg-Arg-p-nitroanilide, Z-Arg-Arg-7-amido-4-methylcoumarin, and Z-Phe-Arg-7-amido-4-methylcoumarin. A proteolytic activity comparable to mammalian cathepsin B was observed. In addition, we analyzed the degradation of human hemoglobin by schistosomal cathepsin B, which has been suggested to be the physiological target of the protease.

The trematode Schistosoma mansoni lives in human blood vessels, causing the parasitic disease bilharziosis. Approximately 200 million people in tropical countries are infected by the helmint. Infection with S. mansoni results in a life-long chronic disease which is marked by increasing tissue damage caused by eggs deposited throughout the body. With 750,000 deaths annually, bilharziosis is the second most deadly parasitic disease after malaria.

Proteases are key components of the pathogenicity of parasites. They facilitate tissue penetration and determine nutritional sources of the parasite within intermediate and human hosts (1). Cathepsin B is the major thiol protease of adult worms of Schistosoma mansoni and may be a valuable target for therapeutic agents.

Cathepsin B (EC 3.4.22.1) belongs to the family of cysteine proteases. On the basis of sequence analysis, cysteine proteases have recently been classified into ERFNIN and cathepsin B-like cysteine proteases (2). Mammalian cathepsins B are lysosomal proteases involved in intracellular protein degradation. In addition, they are believed to play a role in tumor invasion and metastasis (3).

Only little is known about cathepsin B of helminths. The genes of cathepsin B from Haemonchus contortus (4, EMBL accession number M60212), Ostertagia ostertagi (5, EMBL accession number M88503), S. mansoni (6, EMBL accession number M21309), and Schistosoma japonicum (EMBL accession number X70968) and of the free-living nematode Caenorhabditi elegans (7, EMBL accession number M74797) have been determined, but the corresponding enzymes have not been well characterized. Among these enzymes, cathepsin B of S. mansoni has evoked most attention as it is believed to be a key enzyme in the degradation of host hemoglobin (8), and as it is highly immunogenic in man.

Early studies suggested that S. mansoni possesses a protease which specifically hydrolyzes human hemoglobin (9), and two groups reported the purification of a hemoglobinolytic protease (10, 11). The latter group described a cysteine protease with a molecular mass of 32 kDa and a substrate specificity similar to mammalian cathepsin B. In contrast to the lysosomal localization of the mammalian cathepsin B, the schistosomal counterpart is secreted into the gut lumen, which is in line with its possible involvement in parasite nutrition (12). Nevertheless, detailed studies of the protease were impossible due to the unavailability of sufficient amounts of purified protein from the obligate parasitic worm.

The gene of schistosomal cathepsin B was isolated from a cDNA gene bank of adult worms (6), taking advantage of the fact that this protease is highly immunogenic in man. The protease (also termed Sm31) has been suggested as an immunodiagnostic antigen of bilharziosis (13), which is at present diagnosed by laborious examination of feces and urine for eggs.

In view of its participation in host hemoglobin degradation and its potential as a possible component of an immunosassay, several attempts to express active schistosomal cathepsin B have been undertaken in the past.

Cathepsin B of S. mansoni has been expressed as a fusion protein with the amino-terminal region of the RNA replicase of the phage MS2 in Escherichia coli. However, the fusion protein aggregated in the cytoplasm and could only be solubilized with strong denaturants (14). We expressed procathepsin B in its unfused form in E. coli, but the recombinant protein was also found to be insoluble. In addition, cathepsin B has been expressed in insect cells, but the yield of soluble enzyme was too low for purification and enzyme characterization (15).

Recently, we succeeded in expressing cathepsin B in Saccharomyces cerevisiae. Here we report on the construction of a plasmid which allowed efficient expression of procathepsin B. The coding region of the zymogen was fused to the mating factor α secretion signal, and the recombinant protein was secreted in the culture supernatant by the yeast cells. It was purified by taking advantage of a hexahistidine affinity tag which was added to the carboxyl terminus of the protein. The zymogen was subsequently processed to active cathepsin B in vitro by pepsin and characterized enzymatically.
EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs. Radiolabeled DNA was obtained from Amersham. Protease inhibitors were bought from Sigma. The substrates N-benzoyl-L-arginine-p-nitroanilide (Z-Arg-Arg-pNA), N-benzoyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-AMC), N- benzoyl-L-arginine-phenylalanine-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC) were purchased from Bachem and Ni²⁺-NTA agarose was from Qiagen. All reagents were at least analytical grade.

Plasmid Constructions

DNA manipulations were carried out essentially as described by Sambrook et al. (16) using the E. coli strains HB101 and C600 as hosts. Site-directed mutagenesis was carried out by PCR using an automated thermocycler (ATAQ, Pharmacia). The DNA of procathepsin B of S. mansoni was cloned into three expression cassettes.

pUC8-Sm31A—The plasmid pMATA21/S1/H-2 (a gift from E. F. Ernst, Düsseldorf) is a pUC8-based vector carrying the EcoRI-HindIII fragment of mating-factor α from S. cerevisiae (EMBL accession numbers J0340 and X15154) comprising the promoter and the prepropeptide of MFα. The MFα presequence was changed by site-directed mutagenesis to a new S1 restriction site. The plasmid was cut with BamHI and ligated to an EcoRI-restricted PCR fragment derived from the carboxyl-terminal part of Sm31, obtained by partial digestion of the plasmid pEMBleryex2-2 (18) which provides an inducible GAL10/CYC1 hybrid promoter (19), a polycloning site for transcriptional termination and polyadenylation, as well as the two selection markers URA3 and leu2-d. To construct pEMBleryex2-Sm31 (Fig. 1B), an AscI (filled-in)BgIII fragment encoding the preproMFα-procathepsin B fusion was isolated from pUC8-Sm31C and cloned into the SalI (filled-in) and BamHI restrictions sites of the pEMBleryex2 polyclony.

pUC8-Sm31B—The plasmid pMATA21/S1/H-2 (a gift from E. F. Ernst, Düsseldorf) is a pUC8-based vector carrying the EcoRI-HindIII fragment of mating-factor α from S. cerevisiae (EMBL accession numbers J0340 and X15154) comprising the promoter and the prepropeptide of MFα. The MFα presequence was changed by site-directed mutagenesis to a new S1 restriction site. The plasmid was cut with BamHI and Stul and ligated to an EcoRI-restricted PCR fragment derived from the carboxyl-terminal part of Sm31, obtained by partial digestion of the plasmid pEMBleryex2-2 (18) which provides an inducible GAL10/CYC1 hybrid promoter (19), a polycloning site for transcriptional termination and polyadenylation, as well as the two selection markers URA3 and leu2-d. To construct pEMBleryex2-Sm31 (Fig. 1B), an AscI (filled-in)BgIII fragment encoding the preproMFα-procathepsin B fusion was isolated from pUC8-Sm31C and cloned into the SalI (filled-in) and BamHI restrictions sites of the pEMBleryex2 polyclony.

Expression and Purification of Procathepsin B

Cytopreserved competent yeast cells of strain HT393 (ura3, pra1, prb1, prc1, psi1, pre1) were prepared according to Dohmen et al. (20) and transformed with pEMBleryex2-Sm31. Ura⁺ transformants were detected on agar minimal plates (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), 20 mg/ml l-tryptophan, adenine, l-histidine, l-methionine, and l-lysine, 30 mg/ml l-leucine) grown at 30°C for 3 days and subsequently cultured on agar minimal plates. For large-scale expression of procathepsin B, 20–200 ml of minimal medium without uracil and leucine were inoculated with transformed yeast cells and grown for 24 h on an orbital shaker. Expression was induced by inoculating the preculture into 10 volumes of complete medium (2% galactose, 1% yeast extracts (Difco), 2% tryptone (Difco), 100 mM sodium phosphate, pH 6.0). These shake-flask cultures were grown for 72 h at 30°C, 100 rpm.

The cleared culture supernatant was brought to 0.3 M NaCl with 5 M NaCl, diluted with 1 volume of buffer A (50 mM sodium dihydrogen phosphate, 300 mM NaCl) and adjusted to pH 8. Then, 0.02–0.002 volume of Ni²⁺-NTA agarose previously equilibrated with buffer A was added and the suspension was stirred overnight at 4°C. The agarose beads were collected by vacuum filtration and washed twice with 0.05 volume of buffer A and twice with buffer B (same as buffer A, but adjusted to pH 7). The matrix was poured into a 10-column or a 26-column (Pharmacia Biotech Inc.), and proteins were eluted with a pH step gradient (buffer A adjusted to pH 6, pH 5, pH 4, and pH 3, flow rate: 1 column volume/h). Procathepsin B eluted at pH 4. Alternatively, procathepsin B was eluted with 100 mM EDTA, 50 mM sodium phosphate, pH 6.3.

SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE according to Laemmli (21) or according to Schägger & von Jagow (22). The gels were stained with Coomassie Blue or electroblotted (Fast-Blot, Bio versatility, Göttingen, Germany) onto nitrocellulose membranes (23) onto polyvinylidene difluoride membranes (23) and stained with Coomassie Blue. After transfer, the membranes were blocked for 30 min with 1% Tween 20 in Tris-buffered saline (TBS), incubated for 1 h with polyclonal anti-procathepsin B rabbit serum diluted 1:2000 into TBST (TBS, 0.05% Tween 20), washed with TBS, and incubated for 1 h with rabbit anti-goat antibodies coupled to alkaline phosphatase (Jackson Immunoresearch Laboratories, 1:5000 in TBST). The membrane was washed again in TBS and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Amino-terminal Sequencing of Proteins

After separation by SDS-PAGE, proteins were blotted onto polyvinylidene difluoride membranes (23) and stained with Coomassie Blue. Bands of interest were cut and used directly for determination of amino-terminal amino acid residues with an Applied Biosystems model 477A protein Sequence. Peptides (digestion fragments of human hemoglobin) were sequenced after HPLC purification.

Determination of Procathepsin B

Due to the lack of enzymatic activity of procathepsin B, the recombinant protein was detected by Western blotting, and the concentration was estimated from Coomassie Blue-stained gels of yeast culture media concentrated by trichloroacetic acid precipitation. The concentration of purifiedzymogen was determined according to Bradford (24) using bovine serum albumin as standard.
Activation of Procathepsin B

1 volume of procathepsin B solution (0.05–0.2 mg/ml) was combined with 0.5 volume of pepsin solution (5–20 μg/ml in 0.5M sodium phosphate, pH 3.0) and incubated at 37°C for 10 to 60 min. The activation reaction was stopped by addition of 9 volumes of assay buffer (pH 6.0) or by adding pepstatin A to a final concentration of 1 μM.

Enzyme Assay

Z-Arg-Arg-pNA—The method of Hasnain et al. (25) was used. Hydrolysis of Z-Arg-Arg-pNA (ε<sub>405</sub> = 10,400 M<sup>-1</sup> cm<sup>-1</sup>) was monitored with a Hitachi U-3000 photometer or with a Bio-Rad UV-3550 microtiter-plate photometer equipped with a 405 nm filter and controlled by the Kinetic Collector Software (Bio-Rad).

AMC Derivates—The hydrolysis of Z-Arg-Arg-AMC and Z-Phe-Arg-AMC was determined according to the methods of Barrett and Kirschke (44) and Barrett et al. (52) which were modified slightly. The stock buffer was 60 mM MES, pH 5.9, 600 mM NaCl, 4 mM EDTA. Each assay tube contained 0.375 ml of stock buffer, 0.1 ml of 30 mM DTT, and 0.95 ml of 0.1% Brij 35. 37.5 μl of enzyme solution (approximately 2 pmol) were added, preincubated 2 min at 37°C, and equilibrated to room temperature. The reactions were started by the addition of 37.5 μl of 4 mM substrate solution in dimethyl sulfoxide and stopped, after an exactly 15-min incubation at 25°C, with 1.5 ml of 100 mM sodium monochloroacetate in 100 mM sodium acetate, pH 4.3. Fluorescence was determined by a fluorescence spectrometer (Model 3000, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, United Kingdom) with the excitation wavelength at 370 nm and emission measured at 460 nm.

Reduction of Methemoglobin

Reduction and oxygenation of commercial methemoglobin (Sigma) to oxyhemoglobin was performed in the cold as follows. Adapted from the procedure of Dixon and Meltosh (53), a column of 70 × 5 mm (Pasteur capillary pipette), filled with Sephadex G-25, was equilibrated with an oxygen-saturated buffer, containing 10 mM MES, pH 6.5, and 1 mM EDTA. 75 μl of a freshly prepared 10% (w/v) solution of sodium hydroxysulfite in this buffer was run on the column and drained into the gel with 50 μl of the equilibration buffer. 100 μl of a saturated aqueous solution of methemoglobin was applied to the column and run in the
The fractions containing the eluate at pH 4.0 were pooled (approximately 100 U–60% in 45 min) and a flowrate of 200 μl/min at 45°C. About 1 nmol of digested cathepsin B-digested hemoglobin was applied on the column and monitored at 220 nm. For preparative runs, about 10 nmol of digested hemoglobin was applied, and individual peak fractions were collected manually.

Isolation of Peptides

Peptides were separated by reversed-phase HPLC through a Vydac (Hesperia, CA) C4, 30-nm narrow bore (2.1 mm) column using 0.1% (v/v) aqueous trifluoroacetic acid with an acetonitrile gradient (0–60% in 45 min) and a flow rate of 200 μl/min at 45°C. About 1 nmol of cathepsin B-digested hemoglobin was applied on the column and monitored at 220 nm. For preparative runs, about 10 nmol of digested hemoglobin was applied, and individual peak fractions were collected manually.

Mass Spectrometry

Peptide masses were determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry on a Vision 2000 (Finnigan MAT, Bremen, FRG) equipped with a nitrogen laser. For each analysis, 1 μl of a reversed-phase HPLC fraction (2–5 pmol of peptide) was mixed with 1 μl of 2,5-dihydroxybenzoic acid as matrix directly on a sample target. Spectra were composed of 10–20 laser shots and calibrated externally with angiotensin and insulin.

RESULTS

Expression of Procathepsin B—Our attempts to express active cathepsin B in E. coli failed due to aggregation of the recombinant protein in the cytoplasm. Therefore, we decided to express procathepsin B as a secretory protein in yeast. We constructed several yeast expression plasmids, but most of them resulted in disappointingly low yields of recombinant protein. Expression of procathepsin B using the constitutive MFα promoter on a yeast episomal plasmid resulted in approximately 20 μg of recombinant protein per liter of culture. In addition, analysis was hampered due to heterogeneous hyperglycosylation of the recombinant protein. Digestion with endo-glycosidase F/N-glycosidase F (Boehringer Mannheim) was necessary to identify an immunoreactive band on Western blots (data not shown).

In a second attempt, a glycosylation site of procathepsin B was destroyed. The primary structure of procathepsin B contains two consensus sequences for N-linked glycosylation, Asn-X-Ser/Thr/Cys (26). However, one of the consensus sequences is followed by a proline residue which often prevents N-glycosylation (27). The native protein is most probably not modified at this site, as only one N-linked sugar chain has been determined experimentally (28). The mutated recombinant protein was no longer glycosylated, but the expression rate of procathepsin B under the control of the constitutive MFα promoter remained low.

Reducing the copy number of constitutive expression units can lead to an increase of secretion efficiency in yeast (29). Our attempts to improve expression using an integrating expression vector or an autonomously replicating expression plasmid, however, resulted in a decrease of expression. High level expression (up to 10 mg/liter) was finally achieved by expressing the propreMFα–procathepsin B fusion protein under the control of the inducible galactose promoter of the yeast episomal plasmid.
mid pEMBLyex2 (Fig. 1). Expression was found to be optimal when the yeast strain HT393, which is deficient in several proteases, was grown in complete medium, thus favoring high biomass accumulation. In Fig. 2A, the culture supernatant of HT393 (pEMBLyex2-Sm31) is compared with the supernatant of a control culture. A dominant 40-kDa protein is present in the culture medium of the expressing yeast strain, but not of the control culture. This protein corresponds to procathepsin B as demonstrated by the Western blot (Fig. 2A). In addition to the 40-kDa band, a protein of about 20 kDa is seen in the Western blot. This protein probably presents a degradation product of procathepsin B (intCb); lane 2; 10 pmol of pepstatin-activated cathepsin B (pepCb); lane M, molecular mass standard.

To accomplish secretion of procathepsin B, the gene was cloned behind the preproMF α peptide. This sequence promotes secretion of the mating factor α in yeast cells. Processing by the KEX2-protease has been reported to be a rate-limiting step in secretion (30). In order to best mimic the authentic KEX2-processing site (Lys-Arg Glu-Ala), the Glu-Ala dipeptide which belongs to the signal sequence of preprocathepsin B was not deleted during construction of the fusion protein. Indeed, the fusion proMF α-procathepsin B was never observed in the culture supernatant of transformed yeast cells.

Purification of Secreted Recombinant Procathepsin B—The addition of a hexahistidine affinity tag to the carboxyl terminus of procathepsin B enabled a purification using Ni\(^{2+}\)-chelate affinity chromatography introduced by Hochuli et al. (31) for the purification of recombinant E. coli proteins. We adapted this chromatographic method to the purification of recombinant histidine-tagged proteins secreted into the culture supernatant. The culture supernatant was batch-adsorbed onto Ni\(^{2+}\)-NTA agarose, which was subsequently loaded onto a column and eluted by applying pH steps (Fig. 2B). Alternatively, the procathepsin B can be eluted from the column using the competitor imidazole or the elutor EDTA.

The eluant of the metal chelate chromatography depended on the elution method applied (Fig. 2C). When using EDTA to desorb the protein from the matrix, a single protein of about 40 kDa (theoretical molecular mass of procathepsin B, 38 kDa) was observed. When the protein was eluted by low pH, two immunoreactive proteins (40 kDa and 35 kDa) appeared in the eluant. The 35-kDa protein was absent when the thiol protease inhibitors and then assayed with Z-Arg-Arg-pNA. Average and standard deviation of three determinations are given. Pepstatin A is an aspartic protease-specific inhibitor. Bestatin inhibits aminopeptidases, and phenylmethylsulfonyl fluoride (PMSF) reacts with the active site of serine proteases.

**TABLE I**

| Inhibitor          | Concentration | Inhibition |
|--------------------|---------------|------------|
|                    | μM           | %          |
| Thiol protease inhibitors |               |            |
| E-64               | 1.3          | 100        |
| Antipain           | 6.7          | 96 ± 0.9   |
| Thiol and serine protease inhibitors |               |            |
| Leupeptin          | 10           | 99.7 ± 0.1 |
| Chymostatin        | 67           | 97 ± 0.5   |
| TLCK\(^{a}\)        | 67           | 96.8 ± 1.5 |
| TPCK\(^{b}\)        | 67           | 94.7 ± 1.6 |
| Other inhibitors   |               |            |
| Pepstatin A        | 10           | 7.3 ± 10.1 |
| Bestatin           | 6.7          | 13 ± 5.8   |
| PMSF               | 1300         | 211 ± 3.0  |

\(^{a}\)TLCK, 1-chloro-3-tosylalano-7-amino-2-heptanone.
\(^{b}\)TPCK, tosylphenylalanyl chloromethyl ketone.
Since no vacuolar cysteine protease has been detected in S. cerevisiae and since the yeast strain used for expression is deficient in a subunit of yscE, the only known cellular cysteine protease of S. cerevisiae, the 35-kDa protein is probably an autoprocessing product of procathepsin B. It is noteworthy that autoprocessing has also been described for mammalian cathepsin B (34, 35) and for papain (36).

Structural Analysis—The amino termini of the recombinant procathepsin B (proCB) and of the 35-kDa protein were determined by amino acid sequencing. The amino terminus of procathepsin B was found to be heterogeneous. Depending upon the preparation, the major amino-terminal residues were Glu-1 or Val-6. In addition, two minor products with the amino-terminal amino acids His-3 and Asn-8, respectively, were detected (Fig. 4). The occurrence of products lacking two amino-terminal residues will be discussed below. On the other hand, the 35-kDa protein had a uniform amino terminus beginning with Gly-49. The molecular mass of this protein, subsequently termed intermediate cathepsin B (intCB), was calculated to be 32 kDa.

In comparison with the native protein, the recombinant zymogen has a ragged amino terminus, is not glycosylated, due to the engineered mutation Asn-183 \( \rightarrow \) Gln, and has six additional carboxyl-terminal histidine residues. These carboxyl-terminal residues are not removed during expression when using the protease-deficient strain HT393. This is demonstrated by the fact that metal-chelate purification was feasible with this strain.

Enzymatic Analysis—Strikingly, neither the zymogen nor intermediate cathepsin B was enzymatically active with small synthetic cathepsin B-specific substrates such as Z-Arg-Arg-pNA or Z-Lys-ONp. The lack of enzymatic activity could not be explained by the presence of an inhibitory propeptide, since the propeptide (calculated molecular mass 5.6 kDa) was not detectable in SDS-Tricine peptide gels. An inhibitory propeptide \( (K_i = 0.4 \text{ nm}) \) has been observed for rat cathepsin B (37).

In order to convert the inactive procathepsin B into an enzymatically active form, several proteases were tested. The aspartic protease pepC, found to activate procathepsin B in a time- and dose-dependent manner (Fig. 5A), SDS-PAGE analysis of the activated protease revealed that a 34-kDa product only slightly shorter than autoprocessed intCB was the enzymatically active species (Fig. 5B). The amino terminus of this product, subsequently termed pepCB, was determined (Fig. 4) and revealed a pepC digestion site only nine residues carboxyl-terminal to the autocatalytic cleavage site. The cleavage by pepC occurs carboxyl-terminal to leucine, which is in line with the substrate specificity of pepC. Interestingly, the removal of only nine amino acids, as compared to inactive intCB, is sufficient for activation.

The pH dependence of pepC-activated schistosomal cathepsin B was studied. It shows a roughly bell-shaped activity profile under nonsaturating substrate concentrations with a pH optimum around pH 6.0 (Fig. 6). As expected, schistosomal cathepsin B is susceptible to all thiol protease inhibitors tested (Table I).

A detailed comparison with native schistosomal cathepsin B is impossible since the native enzyme is only poorly characterized and doubts about the purity of the preparations persist. Lindquist et al. (10) reported a very low \( K_m \) and \( k_{cat} \) values which are more than one order of magnitude higher than the previously published kinetic data on schistosomal cathepsin B. However, the enzymatic properties of the recombinant schistosomal cathepsin B are in line with the better characterized mammalian cathepsin B (Table II). Interestingly, schistosomal cathepsin B seems to prefer Z-Arg-Arg-AMC, whereas rat and human cysteine proteinases prefer the Phe-Arg derivate. Homology of primary structure between schistosomal cathepsin B and mammalian counterparts and the comparable kinetic data as well as similar hemoglobin digestion patterns (Fig. 7) suggest that the recombinant cathepsin B is functionally equivalent to the native enzyme. In addition, recombinant cathepsin B from rat, expressed and secreted by S. cerevisiae, as well as human cathepsin B, expressed in E. coli, renatured, and pepC-activated, were both fully functional (25, 45).

Active site titration with the inhibitor E-64 revealed that pepC-activated recombinant cathepsin B was 60%active (data not shown). The inactive fraction can be explained by denaturation during fermentation and purification or by incomplete pepC activation.

Schistosomal cathepsin B was originally described as a hemoglobinolytic protease isolated from the gut of S. mansoni. Although hemoglobin degradation was considered to be the physiological role of this protease, there are no reports to our knowledge concerning the degradation products of hemoglobin proteolysis.

We analyzed the peptide fragments of hemoglobin digestion by schistosomal cathepsin B. Peptide fragments were separ-

| Protease                      | Substrate          | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
|------------------------------|--------------------|--------------|-----------|------------------|
| Cysteine protease, SMw32*    | Z-Arg-Arg-AFC      | 0.045        |           |                  |
| Adult thiol proteinase, S.m. | Z-Arg-Arg-AMC      | 0.049        | 0.0054    | 9.083            |
| Cathepsin B, rat liver**     | Z-Arg-Arg-pNA      | 8.3          | 1.0       | 8,310            |
| Cathepsin B, human kidney†   | Z-Arg-Arg-AMC      | 58           | 1.3       | 46,370           |
| Cathepsin B, S. m. (recombinant) | Z-Arg-Arg-AMC     | 81           | 0.28      | 297,900          |
|                             | Z-Ph-Ph-AMC        | 129,000      |           |                  |
|                             | Z-Ph-Ph-AMC        | 359,000      |           |                  |
|                             | Z-Arg-Arg-pNA      | 3.6 ± 0.42   | 1.9 ± 0.14| 1,890            |
|                             | Z-Arg-Arg-AMC      | 49 ± 2.7     | 0.3 ± 0.016| 162,000          |
|                             | Z-Ph-Ph-AMC        | 11.3 ± 0.6   | 0.26 ± 0.018| 43,400          |

| a Calculation based on the specific activity 5178 nmol/h/mg (8).  
| b Data from Lindquist et al. (10).  
| c Data from Hasnain et al. (25).  
| d Data from Kuhelj et al. (45).  

## Table II

Kinetic constants of native and recombinant schistosomal cathepsin B and of mammalian cathepsin B

The \( K_m \) values of recombinant and native schistosomal cathepsin B were calculated by linear regression of the Lineweaver-Burk plot assuming a constant error in velocity. Z-Arg-Arg-pNA concentration was in the range 50 \( \mu \)M to 4 mM; substrate inhibition occurred above 4 mM. Z-Arg-Arg-AMC concentration was varied between 20 \( \mu \)M and 800 \( \mu \)M, and Z-Phe-Arg-AMC concentration between 10 \( \mu \)M and 400 \( \mu \)M (substrate inhibition at 400 \( \mu \)M). \( k_{cat} \) was calculated from the slope of an active site titration with E-64 (44). \( r^2 \) was 0.97 or better in all experiments. -AFC, -7-amido-4-trifluoromethylcoumarin; S.m., S. mansoni.
The aim of this study was the functional expression of schistosomal cathepsin B. This protease might play a key role in the nutrition of the parasitic worm and is highly immunogenic in man.

Functional expression of this important enzyme was unsuccessful in E. coli, but secretion by the host organism S. cerevisiae led to active enzyme. This is in line with former observations that secretion can be essential for correct folding and disulfide bridge formation of proteins which naturally pass through the secretion pathway (38, 39). The expression/secretion of procathepsin B was optimized by using an inducible promoter and by increasing the number of expression units.

Purification using metal-chelate chromatography turned out to be extremely simple. There was no need to lyse cells, and the application of the highly specific adsorbent circumvented a volume reduction step usually required to purify secretory proteins.

Although the fusion protein proMF-procathepsin B was processed completely by the prohormone-processing enzyme KEX2, the recombinant protein displayed microheterogeneity at the amino terminus (Fig. 4). Occurrence of the minor product with the amino-terminal residue His-3 can be explained by partial processing with STE13, an aminodipeptidylpeptidase which removes the spacer peptide Glu-Ala-(Glu/Asp)-Ala-Glu-Ala in three steps during processing of mating factor α (40). So far, we have no explanation for the appearance of the products with the amino-terminal amino acids Val-6 and Asn-8.

When comparing the primary structure of mammalian cathepsin B with schistosomal cathepsin B, it is evident that the amino acids of the mature enzymes are well conserved (50-60% identity) whereas those of the propeptide are more divergent (20-30% identity). Clearly, the mature enzyme has more structural restraints than the activation peptide. We also found that the enzymatic properties of rat cathepsin B and schistosomal cathepsin B are comparable.

On the other hand, we observed that the processing mode of the respectivezymogens differs. According to Koelsch et al. (41), there are three processing modes for zymogens: complete self-processing, partially assisted processing, and fully assisted processing. Rat cathepsin B is capable of complete self-processing (34). Although we cannot completely rule out the possibility that a contaminating thiol protease is responsible for the appearance of intermediate schistosomal cathepsin B, our experimental evidence indicates that processing of schistosomal procathepsin B is partially assisted in vitro. In fact, despite numerous attempts under varying conditions, we did not succeed in obtaining active cathepsin B without an assisting...
protease.

In the case of aspartic proteases, the processing mode can be predicted to a limited extent from the primary structure (41). Comparing the amino acid sequences of the propeptides, there is no obvious relationship between the processing site of mammalian cathepsin B (Gly/Met/Ala) and the spatial organization might be important. However, the position of the processing sites differ by 19 amino acids. The mechanism of autoprocessing of mammalian cathepsin B is not understood, but kinetic data with propanapin and human procathepsin B suggest that both intramolecular and intermolecular proteolysis takes place in vitro (35, 36).

As the intermediate schistosomal cathepsin B is not active, it probably does not contribute to the conversion of procathepsin B. Rather, procathepsin B may undergo intramolecular processing, or minor amounts of mature cathepsin B (although not detected in enzyme assays) may catalyze the conversion. Future constructions of hybrid proteins and the introduction of specific amino acid changes in the propeptide of cathepsin B will shed light on the mechanism of zymogen activation and on the underlying structural requirements.

Upon incubation of procathepsin B or intermediate cathepsin B with pepsin, active enzyme was obtained. Since intermediate and pepsin-activated cathepsin B differ only by nine residues, it is tempting to speculate that the two adjacent arginines located within the removed peptide (Fig. 4) interact with the substrate binding site and block the enzyme as long as the peptide is covalently linked to the enzyme.

Our results prove that schistosomal cathepsin B cleaves human hemoglobin at several positions. We were able to determine some of the positions. A dicarboxypeptidase activity has been reported for cathepsin B (46). Although our data did not give any hints that an exopeptidase activity was present, we only used the amino-terminal ends of fragments for the calculation of the consensus sequence of cleavage sites (Table I). The consensus sequence 6X1/181 revealed a low specificity toward globin. We are currently trying to better define the substrate specificity of schistosomal cathepsin B.

The early studies from Senft and co-workers (50, 51) suggested the existence of a protease which prefers hemoglobin toward globin. This result was taken as evidence that this protease is able to degrade human hemoglobin very specifically and effectively and was subsequently termed hemoglobinase. Cathepsin B is not inhibited by phenylalanine (not shown), a property attributed to the hemoglobinolytic activity analyzed by Senft and co-workers (50, 51), and does not show a preference toward hemoglobin. Therefore, cathepsin B does not merit the term hemoglobinase and most likely is not identical with these early reports of hemoglobinolytic activity.

Cathepsin L and cathepsin D have also been proposed to play a crucial role in hemoglobin digestion (47, 48) but so far hemoglobin degradation by these proteases has not been studied in detail.

Hemoglobin digestion by the intraerythrocytic parasite Plasmodium falciparum has been analyzed more thoroughly (42, 49). After the initial and specific attack of a hemoglobinolytic aspartic protease, hemoglobin molecules are further broken down by another aspartic protease and a cysteine protease. The three proteases are reported to act in a synergistic manner. Interestingly, the cysteine protease involved also prefers globin before hemoglobin. However, at this time, it is too early to speculate if hemoglobin digestion in S. mansoni proceeds analogously to the digestion in P. falciparum.

So far it is unclear how schistosomal cathepsin B is activated in vivo, whether cathepsin B preferentially degrades other human serum proteins, and how blood proteinase inhibitors act on cathepsin B. The procedure described here, however, makes it possible to obtain larger amounts of the zymogen, which will enable further studies to define the physiological role of this protease and its possible use in the diagnosis of bilharziosis.

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#### Table III

| Fragment | Position | Measured mass | Calculated mass | Amino-terminal cleavage site |
|----------|----------|---------------|-----------------|-----------------------------|
| A        | 1–31 (α-chain) | 3196.68 | 3196.57 | VKA/AWG |
| B        | 13–31 (α-chain) | 1972.43 | 1972.14 | TKY/FYP |
| C        | 42–49 (α-chain) | 938.21 | 938.05 | FAT/ILSE |
| D        | 88–105 (β-chain) | 2165.86 | 2165.45 | LVT/LLA |
| E        | 108–137 (α-chain) | 1988.69 | 1988.27 | VKA/AWG |

Consensus 6X1/181
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