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Functional Expression of Human Cathepsin S in *Saccharomyces cerevisiae*

PURIFICATION AND CHARACTERIZATION OF THE RECOMBINANT ENZYME*

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A cDNA encoding the human lysosomal cysteine proteinase cathepsin S precursor has been expressed in yeast using the pVT100-U expression vector containing the α-factor promoter. The procathepsin S gene was expressed either as a fusion protein with the pre-region or with the prepro-region of the yeast α-factor precursor gene. Following *in vitro* processing both constructs gave an identical active mature enzyme with a molecular weight of 24,000. After prolonged cultivation of the cells the recombinant protein is also found as an active proteinase in the culture supernatant. The precursor can be activated *in vitro* at pH 4.5 and 40 °C under reducing conditions. The *in vitro* activated enzyme has a 6-amino acid NH_2-terminal extension when compared with the native bovine enzyme.

The purified enzyme displays a bell-shaped pH activity profile with a pH optimum of 6.5 and p*K* values of 4.5 and 7.8. The isoelectric point of the recombinant human cathepsin S is between 8.3 and 8.6 and about 1.5 pH units higher than for the bovine enzyme. The kinetic data for several synthetic substrates and inhibitors reveal a preference for smaller amino acid residues in the binding subsites S_2 and S_3 of cathepsin S. Like the bovine enzyme, the recombinant human cathepsin S is characterized by a broader range of pH stability (pH 5–7.5) than cathepsins B and L.

Lysosomal cysteine proteinases (cathepsin B, EC 3.4.22.1; cathepsin L, EC 3.4.22.15; cathepsin H, EC 3.4.22.16) are considered to be involved both in the physiological protein breakdown (1) and in pathological degradative and invasive processes (2–5). Normally organisms are protected against accidental release or mistargeting of lysosomal cysteine proteinases by protein inhibitors of the cystatin superfamily (6) which are found both intra- and extracellularly. Further protection is afforded by the instability of most of these cysteine proteinases at neutral pH. In contrast to the cathepsins B and L, the more recently characterized cathepsin S (EC 3.4.22.27) is both stable and highly active at pH 7.0 (7) and can be regarded as a new potential participant in proteinase related disorders.

Cathepsin S is a lysosomally located, single-chain, cysteine proteinase with high endopeptidase activity against proteins including elastin and collagen (7, 8). Its substrate specificity shows some similarities with cathepsin L but it is clearly different in its S_2 subsite specificity (9). The primary structures of bovine and human cathepsin S have been reported (10–12); they are 85% identical and show similarities with cathepsin L.

The expression of the cDNAs of mammalian proteolytic enzymes in microorganisms is a useful method to obtain sufficient quantities of these enzymes and to manipulate their sequences for studies of their mechanism and structure. To date expression has been obtained for the following cysteine proteinases: papain (13), cathepsin B (14), and cathepsin L (15). Here we report the expression of a functional human cathepsin S in *Saccharomyces cerevisiae* and the characterization of the recombinant enzyme.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were obtained from New England Biolabs; DNA polymerase (Klenow fragment) from Bethesda Research Laboratories; T4 DNA polymerase, T4 DNA ligase, and thiopropyl-Sepharose 6B were purchased from Pharmacia LKB Biotechnology Inc. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer. Endoglycosidase F/glycosidase F was purchased from Boehringer Mannheim and used according to the instructions of the manufacturer.

Z[1]-VVR-MCA, Z-FR-MCA, Boc-FFR-MCA, Z-LLR-MCA, Z-FVR-MCA, and Z-VVK-NHO-Nbz[2] were synthesized using standard methods in peptide chemistry as reported elsewhere (9). Z-FA-CHN_2 was purchased from Enzyme System Products. Rat cathepsins L and B were purified as described previously (16, 17).

Plasmid Construction—The yeast expression vector used, YpDC222 (39), is based on a modification of the shuttle vector pVT100-U (18), which contains the α-factor promotor and the part of the gene which codes for the prepro-α-factor. The entire cDNA (11) coding for human cathepsin S (GenBank™ with accession number M90696) including a nontranslated part of the 3′ end (1.2-kb *Eco*RI/blunt ended *Xba*I fragment) was cloned into an *Eco*RI and blunt-ended *Bam*HI site of the vector to create plasmid YpCS1. By site-directed mutagenesis (19) the coding sequence of pro-cathepsin S was brought into the reading frame of the α-factor prepro-sequence to create

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[1] The abbreviations used are: Z-, benzyloxycarbonyl; Boc-, *t*-butyloxycarbonyl; -MCA, 4-methyl-7-coumarylamide; -NBz, 4-nitrobenzoyl, -NHO- is the hydroxylamine moiety; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; PAGE, polyacrylamide gel electrophoresis. For the discussion of the interactions between proteinase and substrate the nomenclature of Schechter and Berger (38) was used.

[2] D. Brömme, U. Neumann, H. Kirschke, and H. U. Demuth, in preparation.

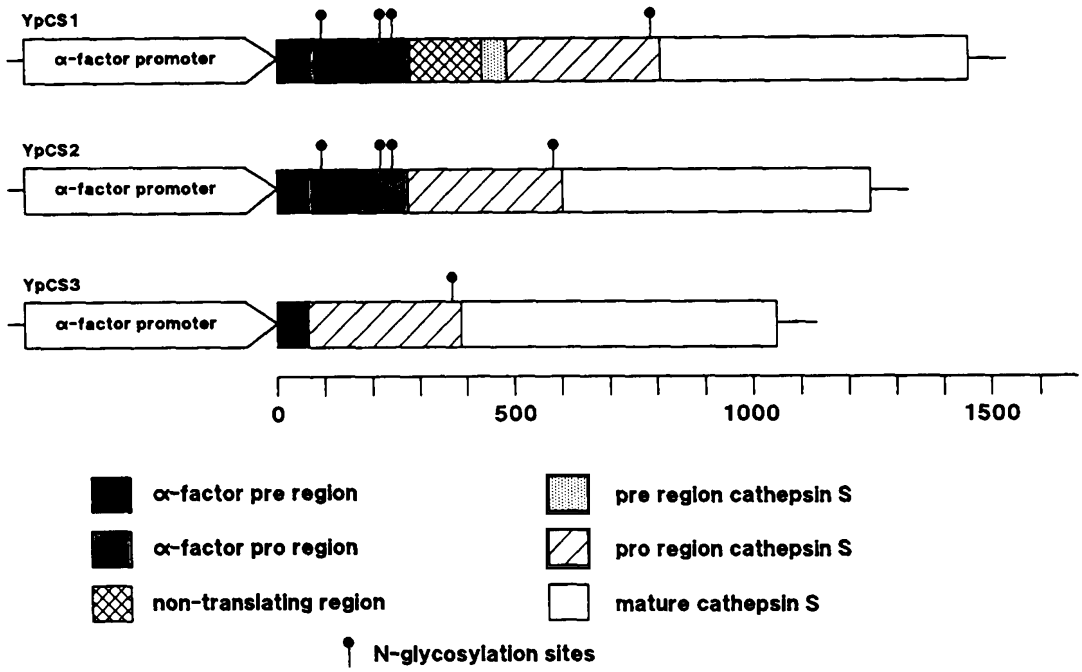

FIG. 1. **Construction of vectors for the expression of the human cathepsin S cDNA in *S. cerevisiae*.** The final expression vectors YpCS2 and YpCS3 were derived from the yeast vector YpDC222, based on the yeast shuttle vector pVT100-U.

plasmid YpCS2 and to the α-factor pre-sequence to create plasmid YpCS3. The following oligonucleotides were used to make these constructions: 5′-AGA GAG GCT GAA GCT CAG TTG CAT AAA GAT-3′ and 5′-GCA TCC TCC GCA TTA GCT CAG TTG CAT AAA CAT-3′, respectively. The replacement of the active-site cysteine residue at position 25 in mature cathepsin S by serine (C25S mutant, designated YpCS4) used the oligonucleotide: 5′-TCT TGT GGT GCT AGC TGG GCT TTC-3′. Plasmid YpCS5 (T-9A) was created to remove the single potential glycosylation site in cathepsin S using the oligonucleotide: 5′-AGA AAT ATC GCA TAT AAG TCG AAC CCT ATT CGG-3′. Restriction enzyme analyses and sequencing confirmed the sequence of the alterations in these plasmids. The most probable cleavage site of the signal peptide of cathepsin S (Ala^{16}-Gln^{17}) was predicted according to von Heijne (20). The probability score for this site was 9.84.

Host Strains and Culture Conditions—Escherichia coli MC1061 was used as the host for most plasmid manipulations. *E. coli* CJ 236 served as the host for the synthesis of single-stranded DNA for site-directed mutagenesis (Bio-Rad Muta-Gene™ Phageamid *in vitro* mutagenesis kit). *E. coli* was grown in 2YT broth (21) at 37 °C with 100 μg/ml ampicillin.

The final expression vectors YpCS2 and YpCS3 were transformed into the yeast strain BJ3501 (22) (Matα pep4::HIS3, prb1-1.6R, his3 Δ200, ura3-52) following the method of Ito *et al.* (23). Transformants were grown for 2 days in 100 ml of synthetic selective medium (24) and then for 4 days in 1 liter of rich medium supplemented with 4% casamino acids, 2% dextrose, and 0.5% ammonium sulfate (production medium) at pH 5.5 and 30 °C.

For the detection of the precursor of cathepsin S the plasmids YpCS3 (wild-type cathepsin S) and YpCS5 (T-9A mutant) were expressed in BJ3501 and YpCS4 (C25S mutant) in BJ3501 and in the yeast strain mnn9 (Matα pep4-3, leu2, ura3-52, mnn9). The tranformants were grown for 2 days in 20 ml of selective medium and then for 4 days in 100 ml of YPD medium at pH 6.5 and 30 °C.

Purification of Recombinant Human Cathepsin S—The yeast cells were harvested from the production medium by centrifugation at 5,000 × *g* and then supplemented with phenylmethylsulfonyl fluoride and EDTA (0.5 and 5 mM, respectively). The cells were lysed by three passages through a French press cell at 20,000 p.s.i. The cell lysate was adjusted to 100 ml of 100 mM sodium acetate buffer, pH 4.5, containing 5 mM dithioerythritol and 5 mM EDTA, and the enzyme was activated at 40 °C for 3 h. Activation was monitored using a fluorogenic substrate assay (substrate: 10 μM Z-VVR-MCA). The activated lysate was clarified by centrifugation at 20,000 × *g*. Ammonium sulfate was added to 80% saturation at room temperature and the enzyme activity collected by centrifugation at 20,000 × *g* was contained in the pellet. The pellet was dissolved in 100 mM sodium acetate (pH 4.0), clarified by centrifugation and the clear supernatant was applied to a 2-pyridyl disulfide-activated thiopropyl-Sepharose 6B column equilibrated with 100 mM sodium acetate (pH 4.0) (25). After washing the column with 60 ml of sodium acetate (pH 4.0), 1 mM EDTA and then with 60 ml of Tris/HCl (pH 8.0), 1 mM EDTA the activity was eluted with 20 mM cysteine, 1 mM dithioerythritol in 100 mM Tris/HCl (pH 6.5), 1 mM EDTA. Electrophoretically homogeneous fractions of recombinant human cathepsin S were obtained by a second passage through activated thiopropyl-Sepharose 6B.

The culture medium supernatant (1 liter) which also contained cathepsin S activity was concentrated using an Amicon Diaflo S1Y10 spiral cartridge to about 60 ml and then to 5–10 ml using an Amicon filter (10PM10) and then applied directly to the activated thiopropyl-Sepharose 6B column. Alternatively, the concentrate was added to the ammonium sulfate precipitate from the intracellular fraction of the cathepsin S preparation.

Protein concentrations were determined by the method of Bradford (26). The active site concentration of the proteinase was determined with E-64 (17). NH_2-terminal sequencing was carried out by automated Edman degradation performed on a model 470A gas-phase sequenator equipped with an on-line model 120A phenylthiohydantoin analyzer (Applied Biosystems Inc.).

Kinetic Measurements—Initial rates of hydrolysis of the methylcoumarylamide substrates were monitored in 1-cm cuvettes at 25 °C in a Cary 2200 spectrophotometer at an excitation wavelength at 380 nm and with a 450-nm emission filter. The kinetic experiments and the processing of the data were carried out as previously described (9). pH profiles were obtained at 2.5 μM substrate (Z-VVR-MCA) concentration ([S] ≪ K_m where the initial rate v_0 is directly proportional to the k_{cat}/K_m value). The following buffers were used for the pH activity profiles: 90 mM sodium citrate (pH 3.0–5.6), 90 mM sodium phosphate (pH 5.8–8.0), and sodium borate (pH 8.0–9.2). All buffers contained 1 mM EDTA and 0.4 M NaCl to minimize the variation in ionic strength. For the least square regression analysis using the Enzfitter program (27) of the pH activity data a four-protonation state model (28) was used for cathepsin L (model 1) and a three-protonation model (28) for cathepsin S (model 2):

$$\text{Model 1: } E(H)_3 \rightleftharpoons E(H)_2 \rightleftharpoons EH \rightleftharpoons E^- \downarrow k_{cat}/K_m$$

$$\text{Model 2: } E(H)_2 \rightleftharpoons EH \rightleftharpoons E^- \downarrow k_{cat}/K_m$$

Proteolytic Digestion of Procathepsin S—Samples of concentrated culture supernatant of YpCS4 (C25S mutant) expressed in BJ3501 cells (500 μg/ml) were incubated with purified mature cathepsin S (0.4 μg/ml) and subtilisin BPN′ (15 μg/ml). The digest with cathepsin

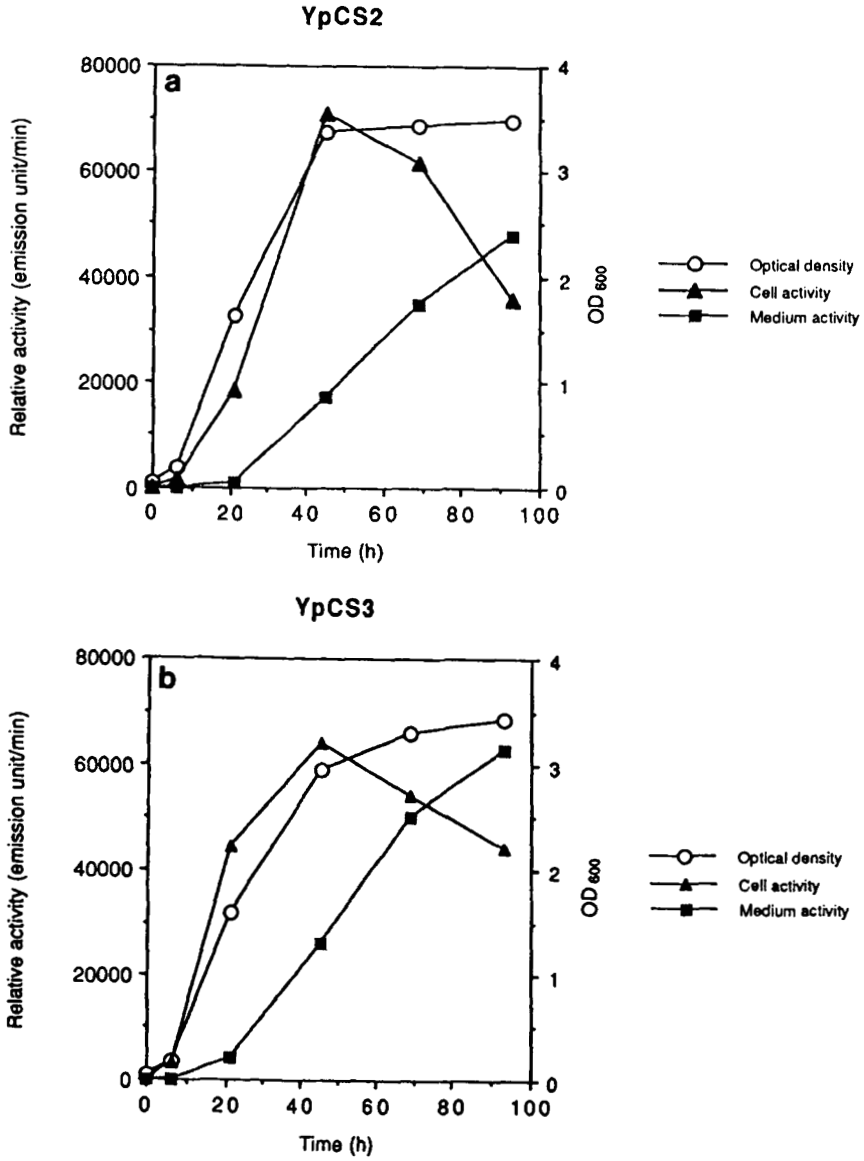

FIG. 2. **Time course of the production of recombinant human cathepsin S in yeast using the vectors YpCS2 (*a*) and YpCS3 (*b*).** The intra- and extracellular activities refer to 1-liter yeast culture and were determined with 10 μM Z-VVR-MCA. The extracellular activity was directly measured from aliquots of the culture medium supernatant and the intracellular activity was determined from cell fractions after *in vitro* activation (see "Experimental Procedures"). The cell samples (1 ml of the culture) were lysed in 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 5 mM dithioerythritol, 0.1 mg/ml zymolase for 2 h at 30 °C and then disrupted with glass beads.

S was performed in 50 mM phosphate buffer (pH 6.5) containing 2.5 mM EDTA and 2.5 mM dithioerythritol and with subtilisin BPN′ in 50 mM Tris/HCl (pH 8.0) at 25 °C. In time intervals aliquots were withdrawn and stopped with 100% trichloroacetic acid (final concentration of 10%). The samples were precipitated with acetone and applied to SDS-PAGE. The visualization was done by immunoblotting.

Immunoblotting and Isoelectric Focusing—SDS-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide and the proteins were transferred to nitrocellulose (29). Detection was carried out with rabbit antibodies raised against ompA-pFlag human procathepsin S (isolated as inclusion bodies from *E. coli* using the expression vector pFlag-1TM (Eastman; data not shown) and horseradish peroxidase protein A conjugate (Bio-Rad) using the ECL detection system from Amersham Corp.

For the analysis of the crude culture medium following clarification and 10-fold concentration, acetone precipitation was performed.

The isoelectric point of recombinant human cathepsin S was determined by Phast Gel IEF 3-9 (Phast System Pharmacia) using the procedure recommended by the manufacturer.

RESULTS AND DISCUSSION

Expression, Activation of Precursor, and Purification of Human Cathepsin S—Yeast BJ3501 Matα cells were transformed with the vectors YpCS2 and YpCS3, coding for procathepsin S fused either with the entire α-factor prepro- or pre-region (Fig. 1). Both constructs gave an identical profile of expression according to the amount of active cathepsin S in the growth medium and to the activatable cathepsin S activity found within the cell (Fig. 2, *a* and *b*). This confirms the results of Ernst (30) that the pro-segment of α-factor is not absolutely required for efficient secretion and processing of proteins.

Cathepsin S was synthesized as an inactive enzyme form within the yeast cell. The precursor could be activated under reducing conditions (2 mM dithioerythritol) at acidic pH. The pH optimum of activation was determined for the enzyme from both plasmid constructs at pH 4.5 (Fig. 3). At pH values below pH 3.5 and above pH 6.0 the rate of activation was about 50–100 times slower. A similar pH dependence was also found for recombinant papain expressed in the baculovirus/insect cell system (31). The optimum temperature of activation of cathepsin S was determined to be 40 °C, and at temperatures above 50 °C the enzyme was inactivated within minutes. For the activation of cathepsin S the following standard conditions were selected: incubation at 40 °C for 3 h at pH 4.5 in the presence of 5 mM dithioerythritol, 5 mM EDTA, and 0.01% Triton X-100.

The precursor of cathepsin S can also be efficiently transformed into the mature enzyme by treatment with subtilisin BPN′. The digest of a crude medium supernatant preparation of the inactive mutant C25S (YpCS4) results in a time-dependent disappearance of the immunoreactive precursor

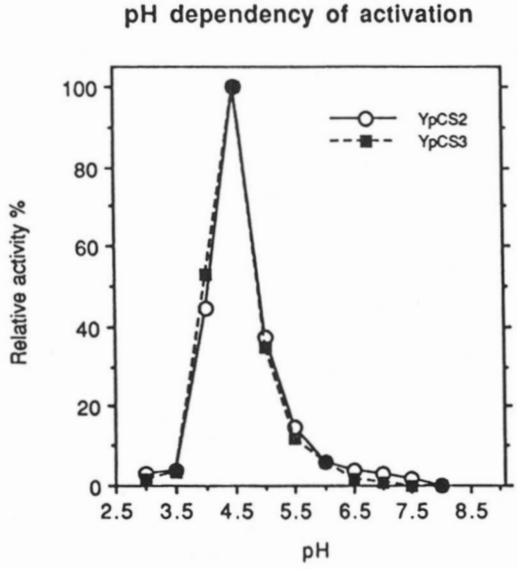

FIG. 3. **pH profile of *in vitro* activation of cathepsin S expressed from YpCS2 and YpCS3 vectors.** The French pressure cell-lysed yeast extract was incubated at 40 °C in the pH range between 3.0 and 8.5 for 3 h. The activity was determined with 10 μM Z-VVR-MCA at 25 °C in 100 mM sodium phosphate buffer (pH 6.5).

TABLE I

Purification of recombinant human cathepsin S[a]

Assay	Total protein	Total activity	Specific activity	Purification factor	Yield
	mg	*μmol/min*	*μmol/min/mg*		%
Crude[b]	352	1,126	3	1	100
$(NH_4)_2SO_4$ precipitate	48	878	18	6	78
Supernatant after pH 4 precipitation	21	844	40	13	75
Thiopropyl-Sepharose 6B					
1	0.21	630	5,250	1,640	56
2	0.05	360	7,200	2,250	32

[a] From 1 liter of production medium (YpCS3 construct).
[b] Pooled intra- and extracellular activity (see "Experimental Procedures").

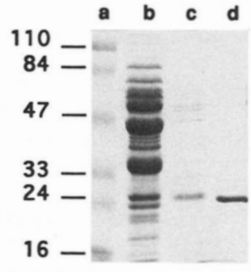

FIG. 4. **SDS-PAGE of purified recombinant human cathepsin S (Coomassie Blue staining).** *Lane b*, $(NH_4)_2SO_4$ fraction; *lane c*, fraction after a single passage through thiopropyl-Sepharose 6B; *lane d*, fraction after a second passage through thiopropyl-Sepharose 6B. Molecular mass standards (kDa) are indicated in the *left margin*.

band and the appearance of the mature enzyme with a molecular weight of 24,000 (see Fig. 5). A digest with purified cathepsin S shows the same pattern on the immunoblot (not shown).

The treatment of the crude, already partially active, wild-type precursor preparation with subtilisin BPN′ results in an 8-fold increase in activity after 1-h incubation at 25 °C. This activity can be completely inactivated by E-64.

Since the intracellular and the extracellular cathepsin S activities were kinetically indistinguishable (data not shown)

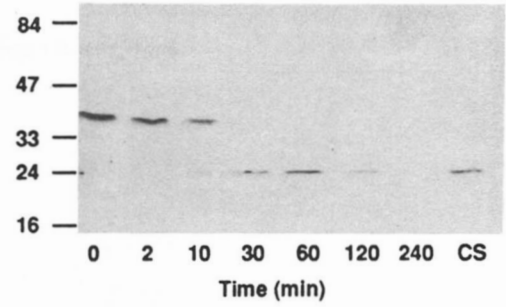

FIG. 5. **Maturation of procathepsin S with subtilisin BPN′.** Aliquots of a crude preparation of procathepsin S [C25S] (concentrated culture medium supernatant; 500 μg of protein/ml) was incubated with subtilisin BPN′ (15 μg/ml) in 50 mM Tris/HCl (pH 8.0), for various times at 25 °C. The incubation was stopped with 100% trichloroacetic acid to give a final concentration of 10%. The times of digestion are as indicated. *CS*, recombinant cathepsin S standard. Molecular mass standards (kDa) are indicated in the *left margin*.

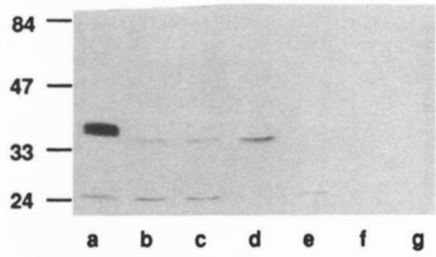

FIG. 6. **Identification of glycosylated and nonglycosylated recombinant human procathepsin S.** Immunoblot of crude preparations (500 μg of protein/ml) of recombinant human cathepsin S precursor (YpCS3, wild type, expressed in BJ3501), glycosylation mutant (YpCS5, T-9A, expressed in BJ3501), C25S cathepsin S precursor mutant (expressed in mnn9) and purified mature cathepsin S. The lanes are: *a*, wild-type precursor in BJ3501 host cells; *b*, wild-type precursor in BJ3501 host cells after endoglycosidase F treatment; *c*, T-9A mutant (procathepsin S with glycosylation site removed) in BJ3501 host cells; *d*, C25S mutant in mnn9 host cells; *e*, recombinant cathepsin S standard; *f* and *g* are crude extracts of BJ3501 and mnn9 host cells bearing the control vector YpDC219 without a coding sequence. Molecular mass standards (kDa) are indicated in the *left margin*.

both activities were pooled to obtain a better yield in the standard purification scheme. By E-64 titration it was determined that about 0.17 mg of cathepsin S was present in 1 liter of the yeast culture after activation and that the final yield of electrophoretically homogeneous enzyme was 30% of that (Table I).

Molecular Weight, Antibody Reaction, NH_2 Terminus, and Immunoelectrophoresis—Purified mature recombinant human cathepsin S expressed in YpCS2 and YpCS3 displays a single band after Coomassie Blue staining in SDS-polyacrylamide gels under reducing conditions and has a molecular mass of 24,000 daltons (Fig. 4). This is in excellent agreement with the M_r of 23,979 based on the cDNA sequence (11). Very recently the expression of human macrophage cathepsin S in COS cells was published (32). The reported molecular mass of 28,000 daltons was derived from a labeling experiment with an iodinated E-64 analogue. The authors (32) assume that this is the molecular weight of the active form of cathepsin S. However, it must be emphasized that a cysteine proteinase precursor or an intermediate of it can also be labeled with E-64 analogues as shown for propapain (31). In contrast, Western blot analysis of preparations of transformed BHK cells (11) as well as transformed yeast cells in this paper (Fig. 5) show clearly a polypeptide with M_r of 24,000. The 24-kDa polypeptide was kinetically characterized and proved similar in all features with bovine cathepsin S (see below).

TABLE II

Comparison of substrate hydrolysis by recombinant human cathepsin S and native bovine cathepsin S

The kinetic constants were determined at pH 6.5 and 25 °C in the presence of 2.5 mM dithioerythritol and 2.5 mM EDTA and represent means ± S.E. The parentheses indicate the number of independent experiments. The concentration of the enzyme was determined by titration with E-64.

Assay	Recombinant human cathepsin S (HCS)			Bovine cathepsin S (BCS)[a]			k_{cat}/K_m	HCS/BCS
	k_{cat}	K_m	k_{cat}/K_m		k_{cat}	K_m		
	s^{-1}	μM	$mM^{-1}\ s^{-1}$		s^{-1}	μM	$mM^{-1}\ s^{-1}$	k_{cat}/K_m
Z-FR-MCA	2.0 ± 0.1	20.8 ± 1.8	96 (2)	YpCS2	4.7	14.7	320	3.8
	1.9 ± 0.2	22.4 ± 2.3	85 (4)	YpCS3				
Z-VVR-MCA	14.4 ± 0.4	17.7 ± 1.5	814 (2)	YpCS2	40.5	17.5	2314	2.8
	15.0 ± 2.1	18.1 ± 2.3	830 (4)	YpCS3				
Boc-FFR-MCA	3.3 ± 0.9	48.0 ± 7.5	69 (3)	YpCS3	8.6	37.5	229	3.3
Z-LLR-MCA	1.1 ± 0.1	3.4 ± 0.1	323 (2)	YpCS3			ND	
Z-FVR-MCA	2.9 ± 0.4	12.1 ± 1.0	240 (2)	YpCS3			ND	

[a] Data from Brömme *et al.* (9).

TABLE III

Comparison of the inactivation parameters of recombinant human cathepsin S and native bovine cathepsin S

The inhibitor studies were carried out at pH 6.5 in the presence of 2.5 mM dithioerythritol and 2.5 mM EDTA at 25 °C and constant enzyme concentration. The values are means ± S.E. for duplicate experiments.

Assay	Recombinant human cathepsin S (YpCS2)			Bovine cathepsin S[a]
	k_2	K_i	k_2/K_i	k_2/K_i
	s^{-1}	μM	$mM^{-1}\ s^{-1}$	$mM^{-1}\ s^{-1}$
Z-FA-CHN2	0.011 ± 0.001	1.25 ± 0.2	8.8	11
Z-VVK-NHO-NBz	0.049 ± 0.005	0.15 ± 0.01	426	545

[a] Data from D. Brömme, U. Neumann, H. Kirschke, and H. U. Demuth, in preparation.

TABLE IV

pK values of pH activity profiles for recombinant human cathepsin S (YpCS2 and YpCS3) and native rat liver cathepsin L

The pK values were obtained from pH activity profiles at 25 °C and constant substrate concentration (2.5 mM Z-VVR-MCA for cathepsin S and 0.5 mM Z-FR-MCA for cathepsin L). The values are means ± S.E. for duplicate experiments.

Assay	pK'_1	pK_1	pK_2	pH optimum
YpCS2		4.47 ± 0.03	7.90 ± 0.03	6.5
YpCS3		4.49 ± 0.03	7.82 ± 0.03	6.5
Cathepsin L	3.33 ± 0.14	4.22 ± 0.28	6.95 ± 0.09	5.5

In contrast to the native enzyme isolated from bovine spleen (12) the mature recombinant human enzyme shows a 6-amino acid NH_2-terminal extension (NH_2-SNPNRI). This was seen in both constructs.

Interestingly, both recombinant cathepsin B (33) and papain (13) are also characterized by an NH_2-terminal extension of 6 and 3–5 amino acid residues, respectively. This may be because the final processing to the mature enzyme is different in yeast and in mammalian cells.

The precursor of human cathepsin S contains one potential site for asparagine-linked glycosylation (N-*X*-S/T) at residue position −11 in the pro-part (11). The molecular weight of the nonglycosylated precursor is 35,900. However, the expression of wild-type cathepsin S in BJ 3501 cells reveals an immunoreactive band of 39–40 kDa (Fig. 6) This higher molecular weight is due to a glycosylation of the precursor, since it binds to concanavalin A-Sepharose. Treatment with endoglycosidase F results in a band which corresponds to 36 kDa. Furthermore, the expression of the mutant T-9A (lacking the single glycosylation site) in BJ3501 and the expression of the inactive C25S mutant in mnn9 cells also reveals an immunoreactive band with a molecular weight of 36,000 in SDS-PAGE (Fig. 6). mnn9 yeast cells are a mutant strain which is defective in polymerization of the outer mannose chain. They are able to synthesize only a core oligosaccharide with 8–13 mannose units (34). This indicates that the proenzyme of cathepsin S is expressed in BJ3501 in its glycosylated form.

Silver staining of isoelectric focusing gels revealed an isoelectric point in the range of 8.3 to 8.6 for the recombinant human enzyme, which is about 1.5 pH units higher than that seen for the native bovine enzyme. This difference in the isoelectric point can be explained by the differing amino acid compositions of the enzymes from the two sources. Taking into consideration the NH_2-terminal extension which carries an additional arginine residue, the human enzyme contains 5 basic residues more than the bovine cathepsin S (R/K to D/E: 25–23 for human cathepsin S and 20–22 for bovine cathepsin S).

Kinetic Analysis of Recombinant Human Cathepsin S—The recombinant human cathepsin S was further characterized using several synthetic substrates and inhibitors and its properties were compared with available kinetic data for the native bovine enzyme (9). The second-order rate constants for the substrate hydrolysis by human cathepsin S and the native bovine enzyme show some relatively small differences. Whereas the pairs of appropriate K_m values for both enzymes are essentially the same, the k_{cat} values are approximately three times lower for the human enzyme (Table II). The second-order rate constants of inactivation for two irreversible inhibitors differ by a factor of 1.3 (Table III). The NH_2-terminal extension of 6 amino acid residues in the recombinant enzyme should not be responsible for the lower k_{cat} values for the human enzyme. It could be shown that similar NH_2-terminal extensions in recombinant papain (13, 35) and cathepsin B (33) do not alter the catalytic properties when compared with the native enzyme of the same species. More likely, these rather small differences may be due to species differences. Comparable differences were reported for cathepsin B and cathepsin L from different sources (1, 9, 35, 36).

The second-order rate constant (k_{cat}/K_m) for the Z-VVR-MCA hydrolysis compared to Z-FR-MCA is about 10-fold higher, and this difference is due to about an 8-fold higher k_{cat} value (Table II). Boc-FFR-MCA and Z-FR-MCA, which both contain bulky aromatic residues in the P2 and P3 posi-

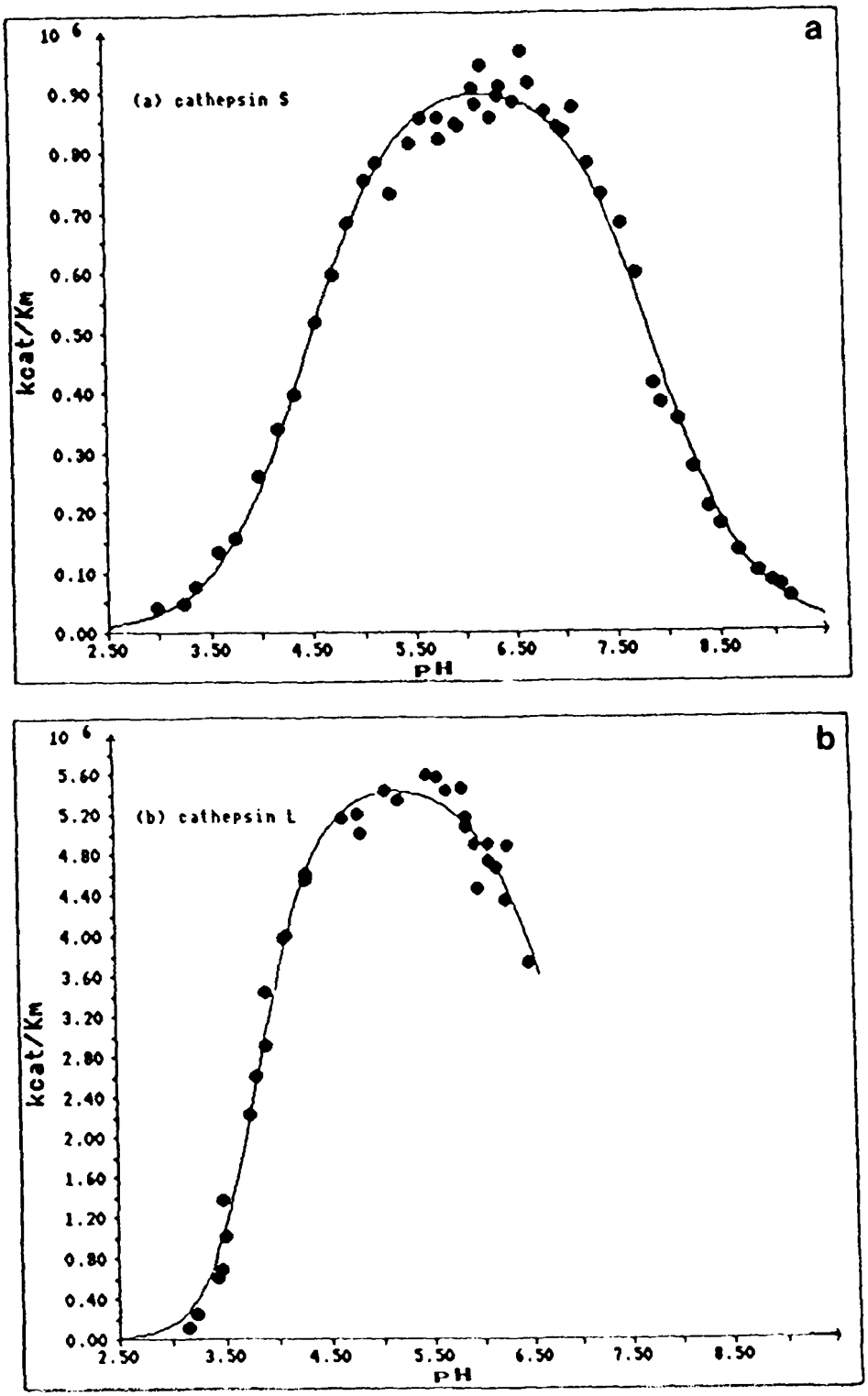

FIG. 7. **pH activity profiles for (*a*) recombinant human cathepsin S and (*b*) rat cathepsin L.** $(k_{cat}/K_m)_{obs}$ values were determined at each pH by measuring the initial rate of Z-VVR-MCA hydrolysis (*cathepsin S*) and Z-FR-MCA hydrolysis (*cathepsin L*), respectively, and by dividing by enzyme and substrate concentration. Data for cathepsin S were best fitted to model 2 and for cathepsin L to model 1.

tions, were hydrolyzed with a similar low efficiency, demonstrating that recombinant human cathepsin S does not prefer neighboring aromatic residues in its S2 and S3 binding subsites. Less bulky hydrophobic residues such as leucine (Z-LLR-MCA) or only 1 aromatic residue in P2 or P3 (Z-FVR-MCA) increase the specificity constant k_{cat}/K_m (Table II). In contrast cathepsin L displays a preference for aromatic residues in both of these positions (9, 37).

The kinetic parameters for the hydrolysis of Z-VVR-MCA and Z-FR-MCA by recombinant cathepsin S are essentially identical for the enzyme from both plasmid constructs YpCS2 and YpCS3 and confirm that both constructs deliver the mature enzyme with the same properties (Table II).

Profiles of pH activity can serve as sensitive measures of enzymatic functional and structural integrity. They are particularly useful for comparison of enzymes of different origins (33). The pH profile of recombinant human cathepsin S from both plasmid constructs (YpCS2 and YpCS3) displays an identical bell-shaped profile with p*K* values of 4.5 and 7.8 (Table IV, Fig. 7*a*) and a pH optimum for Z-VVR-MCA hydrolysis at pH 6.5.

Rat cathepsin L which shares 57% sequence identity with cathepsin S (11) has a different pH profile (Fig. 7*b*). Compared to the pH activity profile for cathepsin S the profile for

TABLE V

pH stability at 37 °C of human cathepsin S in comparison with the rat cathepsins L and B

Recombinant human cathepsin S and the native rat liver cathepsins L and B were incubated at 37 °C in 100 mM sodium acetate buffer, pH 5.0, in 100 mM sodium phosphate buffer, pH 6.5, and in 100 mM Tris/HCl, pH 7.5, respectively. All buffers contained 2.5 mM dithioerythritol, 2.5 mM EDTA, and 0.01% Triton X-100. After 0, 1, 15, and 65 h the activity remaining was determined at 25 °C using 10 μM Z-VVR-MCA as substrate in 100 mM sodium acetate buffer, pH 5.5, for cathepsin L, in 100 mM sodium phosphate buffer, pH 6.0, for cathepsin B and in 100 mM sodium phosphate buffer, pH 6.5, for cathepsin S. The values are means ± S.E. of duplicate experiments.

Assay	Incubation time	Residual activity		
		pH 5.0	pH 6.5	pH 7.5
	hr		%	
Cathepsin S	1	97 ± 1	98 ± 1	93 ± 1
	15	70 ± 2	72 ± 1	49 ± 4
	65	41 ± 2	33 ± 2	7.4 ± 0.3
Cathepsin L	1	99 ± 1	7 ± 1	0
	15	64 ± 2	0	0
	65	5.1 ± 0.5	0	0
Cathepsin B	1	17 ± 2	97 ± 2	0
	15	0	0.5 ± 0.2	0
	65	0	0	0

cathepsin L is shifted toward the acidic range. Above pH 7 cathepsin L is rapidly inactivated and irreversibly denaturated. Two pK values (pK_1′ 3.4 and pK_1 4.2) can be assigned in the ascending limb of the pH activity curve. These values have counterparts in both papain (pK_1′ 3.8 and pK_1 4.3) (28) and in cathepsin B (pK_1′ 3.6 and pK_1 4.9) (33). It remains unclear which of these two values can be related to the active-site cysteine, although in the case of papain the behavior with respect to the variation in the ionic strength has led to the assumption that pK_1 is linked to C-25 (35). An appropriate pK of ~3–4 could not be assigned in cathepsin S. This may be due to the absence of a negatively charged group in the vicinity of C-25 of cathepsin S. A possible candidate is residue 50 (alanine in cathepsin S) which is glutamic acid in papain, cathepsin L, actinidin, and cathepsin H. To clarify this hypothesis site-directed mutagenesis experiments are under way. It is clear that the absence of a pK_1′ is not due to the replacement of Asp^{158} in papain and cathepsin L by Gln^{158} in cathepsin S. As shown by Ménard *et al.* (28) the D158N mutant of papain is best described by a two-ionization process in the low pH range.

Stability of Recombinant Human Cathepsin S—In contrast to the cathepsins L and B, recombinant human cathepsin S is stable for 1 h at 37 °C at pH 5.0, 6.5, and 7.5 (Table V). Native rat cathepsins B and L were completely inactivated after 1 h at pH 7.5. After 15 h of incubation at pH 5 and 6.5 at 37 °C cathepsin S still shows about 70% residual activity and after incubation under the same conditions at pH 7.5 45% residual activity. These data confirm the high stability of recombinant human cathepsin S at neutral and slightly alkaline pH values, a property which has been also demonstrated for the bovine enzyme (7). This stability at neutral pH values indicates that cathepsin S may have an important role in tissue destruction.

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