SUBSTRATE SPECIFICITY OF PROTEINASE *yscA* FROM *SACCHAROMYCES CEREVISIAE*

by

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Proteinase *yscA* is an intracellular aspartic proteinase located in the lysosome-like vacuole of the yeast cell. The specificity towards denatured protein substrates was determined by separation and identification of cleavage products after digestions with proteinase *yscA*, and compared to that obtained with pepsin used under similar conditions. Proteinase *yscA* is more selective towards the peptide bonds it cleaves than pepsin, but shows the same preference for large hydrophobic residues on both sides of the cleaved bond as pepsin and lysosomal cathepsin D. Phe, Leu and Glu are favoured in substrate subsite P_1 and Phe, Ile, Leu and Ala in P'_1 , whereas Val is unfavoured in P'_1 . The implications for the role of proteinase *yscA* as hydrolase maturase are discussed.

1. INTRODUCTION

Proteinase *yscA* is a proteolytic enzyme belonging to the aspartic proteinase family. It is located in the vacuole of the yeast *Saccharomyces cerevisiae*, together with several other hydrolases including two carboxypeptidases, *yscY* and *yscS*, a subtilisin-like proteinase *yscB*, two aminopeptidases, *yscI* and *yscCo*, several phosphatases and a ribonuclease (for review; see ref. 28). The vacuoles of plant cells and fungi share several biochemical and functional features with the lysosomes of the animal cell (18).

Vacuolar aspartic proteinases from fungi and yeasts are not well characterized, in spite of the

increasing interest in yeast as a model system for investigations of eucaryotic cell metabolism. Hence, it was found interesting to analyze the substrate specificity of proteinase *yscA* from *Saccharomyces cerevisiae*.

The primary structure of this proteinase is related to the mammalian aspartic proteinases; with regard to amino acid sequence proteinase *yscA* is 46% identical with porcine lysosomal cathepsin D and 40% identical with porcine pepsin (6). Furthermore, cathepsin D and proteinases *yscA* both have two carbohydrate attachment sites, one of which is in the same position in the two molecules (6, 27). These

Abbreviations: FA = furylacryloyl; LHRH = luteinizing hormone-releasing hormone; RP-HPLC = reverse phase high performance liquid chromatography; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA = trifluoroacetic acid.

Abbreviations of amino acids are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature. The binding site notations for enzymes and substrates are those of SCHECHTER and BERGER (26). Accordingly, the amino acid residues in the amino-terminal direction away from the scissile bond of the substrate are denoted P_1 , P_2 , ..., P_n and in the carboxy-terminal direction P'_1 , P'_2 , ..., P'_n .

structural parallels are consistent with the close relationship between the biochemistry of the lysosomes and vacuoles. In the present study the actions of highly purified proteinase yscA were investigated on unfolded myoglobin, insulin A- and B-chains and ribonuclease, together with the peptides angiotensin II, luteinizing hormone-releasing hormone (LHRH) and substance P and its derivatives.

The results show that proteinase yscA degrades myoglobin at a rate similar to pepsin, though insulin B-chain and oxidized ribonuclease are degraded slower by proteinase yscA than by pepsin. The different extents of hydrolysis reveal that proteinase yscA has a more limited substrate specificity than porcine pepsin. The cleavage pattern of the tested substrates resembles that earlier reported for cathepsin D, extending the previously established relationship between these two enzymes (6, 27).

2. MATERIALS

Proteinase yscA from *Saccharomyces cerevisiae* was obtained by the purification procedure described in reference 6. The resulting preparation was homogenous as judged by SDS-PAGE, and it contained no measurable carboxypeptidase activity, when assayed with the substrate FA-Phe-Leu (4). The molar amount of active enzyme in the preparation was established by titration with the inhibitor pepstatin, using the chromophoric peptide substrate Lys-Pro-Ile-Glu-Phe-(NO₂)Phe-Arg-Leu, kindly supplied by B.M. DUNN (8). Subsequent protein determination by amino acid analysis showed that $95\% \pm 5\%$ of the enzyme was active, based on a molecular weight of 41,500 (6). The activity of proteinase yscA was partially lost during lyophilization, but the enzyme was stable when frozen at -20 °C in 0.05 M ammonium acetate, pH 5.0 in a concentration of 4-20 mg/ml. Since diluted solutions were relatively unstable, freshly prepared solutions were used for each digestion experiment.

Pepsin and the substrates sperm whale myoglobin, bovine ribonuclease, bovine insulin, human angiotensin II, human substance P, substance P (Tyr⁸) and substance P (6-11) were purchased from Sigma, USA. Human LHRH

was a kind gift from CarlbioTech, Denmark. The concentration of pepsin was determined as described above for proteinase yscA. The protein substrates were denatured either by acid treatment (myoglobin) or by performic acid oxidation (5) (insulin and ribonucleases) in order to minimize effects of secondary and tertiary structure. The oxidized A- and B-chains of insulin were separated by gel filtration on BioGel P30 in 50% (v/v) acetic acid, 4 °C, and subsequently lyophilized. All other reagents used were analytical grade.

3. METHODS

3.1. Digestion of substrates

The digestions of protein and peptide substrates with proteinase yscA were normally performed in 0.05 M ammonium formate pH 3.20 at 22 °C or 37 °C. The reaction was initiated by addition of an aliquot of a freshly diluted proteinase yscA solution (0.4 mg/ml) to 2.5 ml thermostated substrate solution. Substrate concentrations ranged from 0.2 mg/ml to 4 mg/ml and enzyme to substrate (E/S) ratios between 1/235 (mol/mol) and 1/23,500 (mol/mol) were used for the various substrates. All the following E/S-ratio's are given in mol/mol. 500 µl aliquots were withdrawn at regular intervals ranging from 2 min to 24 hours. The reaction was stopped by heating in a boiling water bath for 2 min, or by increasing pH to 9.0 with ammonium hydroxide. While proteinase yscA is stable for more than two hours at pH 7.6, 20 °C, it is rapidly and irreversibly inactivated above pH 8.0 (results not shown). The samples were frozen or lyophilized. Digestions of myoglobin and oxidized ribonuclease with pepsin were similarly performed in 0.05 M ammonium formate buffer, but at pH 2.5 where this enzyme is more active. Otherwise the methodology followed the above description for proteinase yscA. Occasionally 3 M urea was included in the substrate solutions. In these cases pH was increased to 4.0 in order to protect proteinase yscA from the denaturing effect of urea at low pH.

3.2. Separation of peptides

Separation of the hydrolysis products was accomplished by high-performance liquid chro-

matography (HPLC) employing Waters HPLC-equipment, as described in reference 6. Solvents used for elution of peptides consisted of 0.1% TFA and gradients were generated with increasing concentrations of acetonitril or n-propanol. Different reverse-phase column materials were employed: Novapak C-18 (Waters Inc., USA), Synchropak wide-pore RP-P (SynChrom Inc., USA) and Vydac wide-pore C-18 (HPLC Technology Ltd., UK). Some peptides co-eluted in the first separation, and rechromatography of the collected fused peaks with different eluent and/or column material was frequently necessary to obtain peptides sufficiently pure for unambiguous identification. The eluents were monitored at 225 nm and 280 nm using a Waters Model 490 detector, recording the former absorption and the ratio. Myoglobin peptides were additionally monitored fluorometrically using a Model 420 fluorescence detector, in order to assign tryptophan containing peptides.

3.3. Analysis of isolated peptides

Peptides were quantified and identified by amino acid analysis on a Durrum D-500 amino acid analyzer. In most cases N-terminal sequencing on either a Beckman 890C spinning cup sequencer for long peptides or an Applied Biosystems gas phase sequencer Model 470A for peptides of small size or in low yield, was used to confirm the identification.

The rates of hydrolysis of different peptide bonds reported here varied widely. Slow cleavages, at rates lower than approx. 1/100 of the highest rates, were disregarded due to the risk of interference from trace amounts of foreign proteases in pepsin or proteinase yscA preparations.

3.4. Kinetic methods

In order to assess the catalytic efficiency of proteinase yscA towards the most labile peptide bond in myoglobin and to compare it with pepsin, rates of hydrolysis of myoglobin were measured as the decrease in peak height following HPLC-separation of aliquots withdrawn during the initial stage of hydrolysis at different substrate concentrations. Hydrolysis rates were ob-

tained at four substrate concentrations for proteinase yscA: 29 μM , 58 μM , 120 μM , and 230 μM (single determinations) and two substrate concentrations for pepsin: 47 μM and 93 μM (duplicate determinations). Rates were measured as mol myoglobin hydrolyzed per second in 1 ml with 1 nM enzyme, based on molecular weights of 41,500 (proteinase yscA) and 35,000 (porcine pepsin). The Michális-Menten parameters, K_m and k_{cat} , together with specificity constants, k_{cat}/K_m , were estimated from intercepts and slopes of double-reciprocal plots of rates vs. substrate concentrations.

4. RESULTS

4.1. Hydrolysis of unfolded protein substrates

Proteinase yscA had very different effects on the denatured protein substrates myoglobin, oxidized ribonuclease and oxidized insulin A- and B-chains. Insulin A-chain proved to be highly resistant to proteinase yscA, since no hydrolysis was detected in samples withdrawn after 300 min of digestion at E/S-ratio 1/1,700. In this respect proteinase yscA differs from rat liver cathepsin D, which cleaves the A-chain at Glu¹⁷-Asn¹⁸ (13). The oxidized B-chain was, however, readily digested, initially producing two fragments. Oxidized ribonuclease was not fully digested even after 24 hours hydrolysis at E/S-ratio 1/1,400. Acid denatured myoglobin proved to be an excellent substrate which was completely degraded at E/S-ratio 1/23,500 after only 10 minutes.

4.1.1. Hydrolysis of acid denatured sperm whale myoglobin.

The first peptide bonds to be hydrolyzed in myoglobin were Leu²⁹-Ile³⁰ and Leu³²-Phe³³. Figure 1a shows the peptide composition after 5 min of hydrolysis at E/S-ratio 1/11,750. Comparable amounts of amino acids were liberated from the peptides Ile³⁰-Gly¹⁵³ and Phe³³-Gly¹⁵³ during Edman degradation on the Beckman-sequencer of the fused peak from HPLC. The peptide Ile³⁰-Arg³¹-Leu³² was eluted in the front peak with the chromatographic conditions employed, but peptide Val¹-Leu³² was never isolated, suggesting that cleavage in myoglobin of Leu³²-Phe³³ occurs subsequent to the removal of

T. DREYER: Specificity of proteinase yscA

Figure 2. RP-HPLC chromatograms of peptide mixtures resulting from digestion of myoglobin (a) and ox. ribonuclease (b) with porcine pepsin. The chromatographic conditions were the same as described in Figure 1 for myoglobin and ox. ribonuclease, respectively.

Val¹-Leu²⁹. However, as large peptides resulting from the initial cleavages proved difficult to separate efficiently, it cannot be ruled out that small amounts of Val¹-Leu³² is eluted under the myoglobin peak during separations corresponding to early stages of hydrolysis, even though amino acid analysis of the myoglobin peak gave no indication of significant contamination. The peptide Val¹-Leu²⁹ is very hydrophobic and late eluting from RP-HPLC columns, and recoveries varied between 0% and 40%, depending on the sample treatment before analysis. The highest recovery was obtained when 3 M urea was included in the myoglobin solution during digestion. Urea apparently prevents precipitation or agglomeration of this peptide during heating

or freezing/thawing. Figure 1b shows the peptide composition after 60 min hydrolysis at E/S-ratio 1/2,350. At this point considerable cleavage of four more peptide bonds was evident: Glu¹⁰⁹-Ala¹¹⁰, Leu¹³⁷-Phe¹³⁸, Leu⁶⁹-Thr⁷⁰ and Phe¹⁰⁶-Ile¹⁰⁷. Further hydrolysis produced several smaller fragments derived from slow cleavages in the N-terminal region: Leu²-Ser³, Gln⁸-Leu⁹, Leu¹¹-His¹², Trp⁷-Gln⁸ and also Ala¹³⁴-Leu¹³⁵.

Pepsin was shown to cleave almost the same peptide bonds in myoglobin as proteinase yscA, although Leu³²-Phe³³ was apparently not susceptible to hydrolysis. In addition, the hydrophobic N-terminal region was more extensively hydrolyzed by pepsin due to cleavage of Leu⁹-Val¹⁰ and Val¹³-Trp¹⁴. Figure 2a shows the fractionation of peptides from digestion with pepsin for 60 min at E/S-ratio 1/2,350. Rat liver cathepsin D is reported by IMOTO et al. (13) to cleave denatured myoglobin in the same positions as shown here for proteinase yscA, except for position Glu¹⁰⁹-Ala¹¹⁰. Cathepsin D is claimed to cleave Ala¹¹⁰-Ile¹¹¹ instead (see Discussion). Small deviations between proteinase yscA and cathepsin D specificities are observed towards slowly hydrolyzed peptide bonds in the N-terminal region of myoglobin. Figure 3 summarizes the comparison of specificities towards myoglobin.

4.1.2. Hydrolysis of the B-chain of oxidized bovine insulin

The B-chain of oxidized insulin was initially cleaved only at Leu¹⁵-Tyr¹⁶ (Fig. 1c). When the E/S-ratio was increased five-fold from 1/57,000 to 1/11,400 and the incubation time doubled from 30 to 60 min, cleavage was also obtained at Phe²⁴-Phe²⁵, Ala¹⁴-Leu¹⁵, Glu¹³-Ala¹⁴ and Tyr²⁶-Thr²⁷ (Fig. 1d). Pepsin has been reported to cleave this substrate primarily at Tyr¹⁶-Leu¹⁷, Leu¹¹-Val¹² and Phe²⁵-Tyr²⁶ (25) which are not cleaved by proteinase yscA, and at Phe²⁴-Phe²⁵.

Figure 1. RP-HPLC chromatograms of peptide mixtures resulting from initial (upper row) and extensive (lower row) digestion of myoglobin (a, b), ox. insulin B-chain (c, d) and ox. ribonuclease (e, f) with proteinase yscA. Peptides were eluted from a Vydac C-18 column (panels a-d) or a Novapak C-18 column (panels e-f) by generating linear gradients of acetonitril in 0.1% TFA, 1 ml/min, in panels a and b: 9%-54% acetonitril in 80 min, in panels c and d: 18%-41% acetonitril in 50 min and in panels e and f: 9%-50% acetonitril in 80 min.

Figure 3. Cleavage sites in denatured protein substrates after hydrolysis with proteinase yscA, porcine pepsin and rat liver cathepsin D (6, 13, 27, 29).

Secondary cleavages occur at Glu¹³-Ala¹⁴, Ala¹⁴-Leu¹⁵ and Leu¹⁵-Tyr¹⁶, in addition to three peptide bonds left untouched by proteinase yscA: Gly²³-Phe²⁴, Phe¹-Val² and Gln⁴-His⁵. The close similarity in the specificity of proteinase yscA and cathepsin D towards myoglobin is not mirrored in the insulin B-chain, as seen from Figure 3. Cathepsin D cleaves Tyr¹⁶-Leu¹⁷ and Phe²⁵-Tyr²⁶, like pepsin, in addition to Leu¹⁵-Tyr¹⁶ (13).

4.1.3. Hydrolysis of oxidized bovine ribonuclease

Oxidized ribonuclease was digested slowly by proteinase yscA even at a high E/S-ratio of 1/1,430 to yield four peptides (Fig. 1e). The cleaved peptide bonds were: Leu⁵¹-Ala⁵², Ser⁸⁰-Ile⁸¹ and Phe¹²⁰-Asp¹²¹. After prolonged incubation (24 hours) the HPLC-peak corresponding to the peptide Lys¹-Leu⁵¹ broadened (Fig. 1f). This is probably due to contamination with the peptide Lys¹-Glu⁴⁹, as sequencing of the mixture of peptides after digestion, but before separation, indicated the presence of a peptide with

N-terminal sequence Ser-Leu-Ala-, as evidence of slow cleavage of the peptide bond Glu⁴⁹-Ser⁵⁰.

Pepsin similarly cleaved oxidized ribonuclease at Leu⁵¹-Ala⁵², Phe¹²⁰-Asp¹²¹ and Ser⁸⁰-Ile⁸¹, but additionally at Thr⁴⁵-Phe⁴⁶ and Gln⁵⁵-Ala⁵⁶ (Fig. 2b and Fig. 3). Glu⁴⁹-Ser⁵⁰, cleaved by proteinase yscA, was apparently resistant to the action of pepsin.

No data were found in the literature on the hydrolysis of this substrate by cathepsin D.

4.1.4. Kinetics of hydrolysis of myoglobin

Table I gives estimates of the Michālis-Menten parameters and specificity constants for the digestion of myoglobin with proteinase yscA and pepsin at pH 3.20 and pH 2.50, respectively, in 0.05 M ammonium formiate, 37 °C. Initial hydrolysis rates of the Leu²⁹-Ile³⁰ peptide bond were obtained at the substrate concentrations described in section 3.4. The differences in kinetic parameters obtained with the present two datasets for proteinase yscA and pepsin are not statistically significant.

4.2. Hydrolysis of peptide substrates

Small synthetic substrates with systematic variations in subsite side chains, developed and used for the characterization of pepsin specificity (9), were either cleaved too slowly or not at all by proteinase yscA (results not shown). Chromophoric octapeptide substrates, designed according to established pepsin subsite specificity by DUNN et al. (8) are cleaved by proteinase yscA between two phenylalanines. The enzyme appears, however, to be among the least active towards this class of substrates, with kinetic constants close to those of bovine cathepsin D and chymosin (7).

Pharmacologically active peptides have been used extensively for the analysis of either specificity or physiological significance of proteases. Hence, the hydrolysis of these substrates with proteinase yscA offers the opportunity for specificity comparisons with other aspartic proteinases. Three peptide hormones and derivatives hereof were chosen as substrates: angiotensin II, luteinizing hormone releasing hormone (LHRH), substance P, substance P (Tyr⁸) and substance P (6-11). The amino acid sequences of the peptide substrates and cleavage sites with proteinase yscA and related aspartic proteinases are shown in Figure 4.

4.2.1. Hydrolysis of angiotensin II

Angiotensin II is an octapeptide hormone resulting from C-terminal removal of the dipeptide -His-Leu from angiotensin I by the peptidase angiotensin-converting enzyme (ACE). Proteinase yscA slowly cleaved the Tyr⁴-Ile⁵ bond of angiotensin II. 55% was cleaved after 5 hours digestion at E/S-ratio 1/2,000, 25 °C, and no other cleavages were observed. Calf chymosin and cathepsin D's isolated from human and rat brains are unable to cleave this substrate (3, 15), whereas fungal extracellular aspartic proteinases from *Mucor*, *Irpex*, *Aspergillus* and *Endothia* species cleave the same Tyr⁴-Ile⁵ bond of angiotensin II (or angiotensin I) (12, 15).

4.2.2. Hydrolysis of luteinizing hormone-releasing hormone (LHRH)

The decapeptide LHRH was highly resistant to the proteolytic activity of proteinase yscA. A small fraction, corresponding to approx. 2% of the substrate was, however, cleaved at the Tyr⁵-Gly⁶ bond when digestion at E/S-ratio 1/3,333 was allowed to proceed for 20 hours at 27 °C.

Chymosin and *Mucor miehei* protease readily cleave this peptide bond in LHRH at E/S-ratio 1/1,700, 3 hours incubation, and the fungal

Figure 4. Hydrolyzed peptide bonds in peptide hormone substrates digested with proteinase yscA and related aspartic proteinases (3, 12, 15). (—: no data found with the enzyme).

Relative rate of hydrolysis	P ₄ P ₃ P ₂ P ₁ P ₁ ' P ₂ ' P ₃ ' P ₄ '	Substrate peptide bond
1.0	- Q - D - I - L - I - R - L - F -	Myo 29-30
1.0	- L - I - R - L - F - K - S - H -	Myo 32-33
0.7	- V - E - A - L - Y - L - V - C -	InB 15-16
0.5	- F - I - S - E - A - I - I - H -	Myo 109-110
0.5	- A - L - E - L - F - R - K - D -	Myo 137-138
0.3	- Y - L - E - F - I - S - E - A -	Myo 106-107
0.2	- V - T - V - L - T - A - L - G -	Myo 69-70
0.2	- L - V - E - A - L - Y - L - V -	InB 14-15
0.2	- P - V - H - F - D - A - S - V -	RNA 120-121
0.1	- E - R - G - F - F - Y - T - P -	InB 24-25
0.1	- H - L - V - E - A - L - Y - L -	InB 13-14
0.1	- H - E - S - L - A - D - V - Q -	RNA 51-52
0.1	- S - T - M - S - I - T - D - C -	RNA 80-81
<0.1	V - L - S - E - G - E -	Myo 2-3
<0.1	- G - E - W - Q - L - V - L - H -	Myo 8-9
<0.1	- Q - L - V - L - H - V - W - A -	Myo 11-12
<0.1	- E - G - E - W - Q - L - V - L -	Myo 7-8
<0.1	- M - N - K - A - L - E - L - F -	Myo 134-135
<0.1	- G - F - F - Y - T - P - K - A -	InB 26-27
<0.1	- F - V - H - E - S - L - A - D -	RNA 49-50

Figure 5. Peptide bonds in myoglobin (Myo), ox. insulin B-chain (InB) and ox. ribonuclease (RNA) cleaved by proteinase yscA. The relative rates of hydrolysis were estimated from quantitative amino acid analysis of recovered peptides after digestion for 60 min at E/S-ratio 1/2,350 and HPLC separation. Hydrolysis rates for insulin B-chain and ribonuclease, obtained at other conditions, were normalized to this E/S-rate and hydrolysis time.

aspartic proteinases from *Irpex lacteus* and *Endothia parasitica* also cleave this bond in addition to His²-Trp³, Gly⁶-Leu⁷ (*I. lacteus*) and Leu⁷-Arg⁸ (*E. parasitica*) (15).

4.2.3. Hydrolysis of substance P and its derivatives

Substance P is an undecapeptide with a Phe⁷-Phe⁸ bond which would typically be subceptible to cleavage by an aspartic proteinase. This bond was, indeed, cleaved by proteinase yscA, in addition to the Gly⁹-Leu¹⁰ bond which was less labile. Two hours incubation at E/S-ratio 1/2,000 resulted in 38% molar conversion of substance P into peptides Arg¹-Phe⁷ (30%), Arg¹-Gly⁹ (8%) and Phe⁸-Met¹¹ (25%). (Numbers in paranthesis are molar recoveries as percentage of mol substrate). Replacement of Phe⁸ with

Tyr⁸ slightly reduced the degree of conversion to 32%, and the peptides generated were Arg¹-Phe⁷ (15%), Arg¹-Gly⁹ (17%) and Tyr⁸-Met¹¹ (14%).

The N-terminally shortened hexapeptide substance P⁶⁻¹¹ under the same conditions was converted 27% into peptides Gln⁶-Phe⁷ (8%), Gln⁶-Phe⁸ (11%) and Gln⁶-Gly⁹ (8%).

Brain cathepsin D and the *Irpex lacteus* proteinase also cleave the Phe⁷-Phe⁸ bond in substance P (3, 15) whereas the *Aspergillus niger* proteinase cleaves Phe⁸-Gly⁹ in substance P and substance P (6-11), and Tyr⁸-Gly⁹ in substance P (Tyr⁸) (12).

5. DISCUSSION

The physiological role of proteinase yscA is controversial. In vitro studies performed in the 1970's suggested that several specific zymogen

activations and enzyme deactivations (e.g. of chitin synthase and enzymes in the gluconeogenic pathway) depended on proteinase yscA (11), but later genetic studies with mutants devoid in proteinase yscA failed to corroborate these results (31). The enzyme was subsequently considered solely a catalyst of unspecific protein break-down, until it was shown that proteinase yscA also acts as a specific maturase for other vacuolar hydrolases (2, 32). How is the catalytic specificity designed to accomplish such diverse functions? The results of the present investigation show that the specificity of proteinase yscA towards denatured protein substrates resembles the specificity of pepsin (23) and particularly of cathepsin D (13), which also has a dual significance, since it is supposed to be involved in protein turnover in the cell as well as specific limited proteolysis associated with inflammatory and malignant processes (10, 16).

Figure 5 summarizes the observed cleavage positions in the three denatured protein substrates, and adjacent residues that may interact with the binding cleft are included. Figure 6 lists the occurrence of side chains in either P_1 or P_1' subsites (SCHECHTER and BERGER notation (26)) of the three substrates tested in this investigation.

It is obvious that proteinase yscA, like pepsin, prefers to cleave peptide bonds joining, mostly large, hydrophobic amino acids, though Ile is limited to P_1' . Figure 7 lists all the peptide bonds with favourable amino acid side chains in P_1 (Leu or Phe) and in P_1' (Phe or Ile) that are not cleaved by proteinase yscA, and it appears that basic side chains in P_1 or P_1' are prohibitive for cleavage by proteinase yscA in analogy with mammalian pepsin, chymosin and cathepsin D. This is in contrast to the extracellular aspartic proteinases produced by the yeast *Candida albi-*

Amino acids in P_1 or P_1'	Number of peptide bonds cleaved	Total number of peptide bonds	Fraction of peptide bonds cleaved
P_1			
Leu	8	24	0.33
Phe	3	12	0.25
Glu	3	21	0.15
Ala	2	31	0.06
Tyr	1	11	0.09
Trp	1	2	0.50
Gln	1	13	0.08
Ser	1	22	0.05
P_1'			
Phe	3	12	0.25
Ile	3	12	0.25
Leu	3	24	0.13
Ala	3	31	0.10
Thr	2	16	0.12
Ser	2	22	0.09
Tyr	1	11	0.09
Gln	1	13	0.08
Asp	1	11	0.09
His	1	18	0.06

Figure 6. Amino acids occupying substrate subsites P_1 or P_1' in peptide bonds of myoglobin, ox. insulin B-chain and ox. ribonuclease, that are cleaved by proteinase yscA.

P ₄	P ₃	P ₂	P ₁	P' ₁	P' ₂	P' ₃	P' ₄	P ₄	P ₃	P ₂	P ₁	P' ₁	P' ₂	P' ₃	P' ₄
- F - K - H - L -	- K - T - E - A -	- T - L - E - K -	- F - D - R - F -	- S - E - D - L -	- K - K - H - G -	- A - A - A - K -	- F - E - R - Q -	- G - A - I - L -	- K - K - K - G -	- T - K - H - K -	- I - P - I - K -	- E - A - E - L -	- K - P - L - A -	- K - F - D - R -	- F - K - H - L -
- F - D - R - F -	- K - H - L - K -	- V - P - V - H -	- F(- D - A - S -)	(- I - R - L -)F -	- K - S - H - P -	- A - N - K - H -	- I - I - V - A -	(- L - E - L -)F -	- R - K - D - I -						
- I - H - V - L -	- H - S - R - H -														
- P - E - T - L -	- E - K - F - D -	- K - Y - L - E -	- F(- I - S - E -)	- I - K - Y - L -	- E - F(- I - S -)	- H - G - Q - D -	- I - L(- I - R -)	- N - K - A - L -	- E - L(- F - R -)	- F - R - K - D -	- I - A - A - K -	- A - A - K - F -	- E - R - Q - H -		
- L - E - K - F -	- D - R - F - K -														
(- L -)T - A - L -	- G - A - I - L -	- G - E - R - G -	- F(- F - Y - T -)	- Y - K - E - L -	- G - Y - Q - G -	- A - L - G - A -	- I - L - K - K -	- P - G - N - F -	- G - A - D - A -	(- I - S - E -)A -	- I - I - H - V -	- L - K - P - L -	- A - Q - S - H -		
- G - S - H - L -	- V - E(- A - L -)			(- A - L -)Y - L -	- V - C - G - E -			(- E - W - Q -)L -	- V - L(- H - V -)						
- V - N - T - F -	- V - H - E(- S -)	(- S - E -)A - I -	- I - H - V - L -												
	F - V - N - Q - H -	- N - H - K - I -	- I - V - A - C -												
(- R - G - F -)F -	- Y(- T - P - K -)	- H - K - I - P -	- I - K - Y - L -												
- S - R - N - L -	- T - K - D - R -	- P - V - N - T -	- F - V - H - E -												
- N - Q - H - L -	- C - G - S - H -	- H - P - G - N -	- F - G - A - D -												
			F - V - N - Q -												

Figure 7. Peptide bonds in myoglobin, ox. insulin B-chain and ox. ribonuclease with Leu or Phe in P₁ (left column) or with Ile or Phe in P'₁ (right column), that are not cleaved by proteinase yscA. Empty positions are due to the N- or C-termini of the substrate molecule and brackets enclose sequences cleaved off by proteinase yscA.

cans and by fungi belonging to *Penicillium*, *Endothia*, *Aspergillus*, *Rhizopus*, *Irpex* and *Cladosporium* genera (12, 14, 15, 24). These enzymes (but not *Mucor* rennin) exhibit a trypsin-like preference for a basic residue in the P₁-position of the substrate, and the enzymes from *Endothia parasitica* and *Irpex lacteus* moreover have been shown to accept arginine in the P'₁ substrate subsite (15). The fungal extracellular enzymes also share a relative distant prima-

ry structure homology with pepsin, cathepsin D and proteinase yscA (25%-30% sequence identity). Some fungi and *Candida* yeasts, however, in addition to the extracellular proteinases, have been shown to produce intracellular (vacuolar) aspartic proteinases, which have been far less characterized (17, 22). It is likely that these enzymes are pepsin-like and that they occur in all fungi, with similar physiological significance and biochemical properties as yeast vacuolar

Table 1. Michaelis-Menten parameters and specificity constants for the hydrolysis of myoglobin by proteinase yscA and porcine pepsin at 37 °C in 0.05 M ammonium formate, and at pH 3.20 and 2.50, respectively.

	K _m (mM)	k _{cat} (sec ⁻¹)	k _{cat} /K _m (mM ⁻¹ sec ⁻¹)
Proteinase yscA	0.08 ± 0.01	3.9 ± 0.8	50 ± 5
Pepsin	0.20 ± 0.01	8.2 ± 3.0	40 ± 3

proteinase yscA and the mammalian lysosomal cathepsin D.

The acid Glu is frequently occurring in P_1 , though Asp is disliked in this position (Fig. 7). Pepsin has a weak preference for both these residues in P_1 (23). Glu is not accepted in P'_1 , but one cleaved bond has an Asp in P'_1 . Pepsin has a high preference for Glu in P_2 which is apparently mirrored in proteinase yscA: 4 out of the 20 positions cleaved have a Glu in P_2 . A dislike for Lys and Arg in P_3 , as in pepsin (23), is probably the reason why Leu⁸⁹-Ala⁹⁰ in myoglobin and Leu³⁵-Thr³⁶ in oxidized ribonuclease are resistant to hydrolysis. There are, however, important differences between pepsin and proteinase yscA. Proteinase yscA dislikes peptide bonds with charged or small amino acid side chains in P'_1 (see Fig. 7) but Val is also prohibitive, whereas pepsin has a positive preference for Val in this position (23). Three peptide bonds in the protein substrates with Val in P'_1 are cleaved by pepsin but not by proteinase yscA (Leu⁹-Val¹⁰ in myoglobin and Phe¹-Val² and Leu¹¹-Val¹² in insulin B-chain). The Tyr⁶-Leu¹⁷ bond in insulin B-chain is cleaved by pepsin and cathepsin D, but not by proteinase yscA. This could result from a dislike for Tyr in P_1 , but more likely from a rapid removal of adjoining peptide segments (Phe¹-Leu¹⁵), leaving too few amino acids for proper enzyme-substrate interactions at the N-terminal side of Tyr⁶-Leu¹⁷.

Finally, an important difference is in the degree of hydrolysis of the substrates. The values of k_{cat}/K_m for hydrolysis of the first peptide bond in myoglobin by proteinase yscA and pepsin suggest a similar catalytic efficiency. Pepsin, however, cleaves 8.9% (27 bonds) of all peptide bonds in the three protein substrates (304 bonds), whereas proteinase yscA only cleaves 6.6% (20 bonds), indicating a narrower specificity. The numbers do not correspond to "terminal" cleavage, but refer to extensive digestions, for which the conditions are described in the Methods section. The statistical cleavage probability of a peptide bond by pepsin has been reported to be 15% after deepgoing digestion used during protein sequencing (23).

In insulin B-chain and myoglobin, cathepsin D has been shown to cleave in 10, or perhaps 11, out of the 16 cleavage positions observed with

proteinase yscA (13). The assignment of cleavage position Ala¹¹⁰-Ile¹¹¹ in myoglobin with cathepsin D was based solely on amino acid analysis of the putative peptide Ile¹¹¹-Leu¹³⁷. The peptide Ala¹¹⁰-Leu¹³⁷, resulting from hydrolysis with proteinase yscA, in our hands yielded ambiguous values for Ala (between 6 and 7) by amino acid analysis. N-terminal sequencing established Ala¹¹⁰ as the N-terminal, and consequently Glu¹⁰⁹-Ala¹¹⁰ as the cleaved bond, and this might also be the case with cathepsin D. The major difference between the substrate specificities of proteinase yscA and cathepsin D towards myoglobin and insulin B-chain is observed in the cleavage of the central hydrophobic sequence of insulin B-chain: Cathepsin D was reported not to cleave Glu¹³-Ala¹⁴ and Ala¹⁴-Leu¹⁵ as proteinase yscA and pepsin do. Except for the cleavage of Glu¹⁰⁹-Ala¹¹⁰ (or Ala¹¹⁰-Ile¹¹¹), cathepsin D exclusively cleaves after large hydrophobic amino acids in myoglobin and insulin B-chain.

The cleavage of peptide substrates by proteinase yscA resembles the cleavage pattern obtained with other aspartic proteinases. Proteinase yscA is, however, apparently more active against both angiotensin II and substance P than cathepsin D, reflecting the observations using protein substrates.

The pepsin-like nature of proteinase yscA is in good agreement with one of the proposed functions of the vacuolar proteinases, which is general breakdown of excessive, aberrant and long-lived proteins (28). Accordingly, proteinase yscA is responsible for normal yeast differentiation during sporulation induced by nutrient deficiency; a process involving highly elevated protein turnover (1, 28). The narrower specificities of proteinase yscA and cathepsin D, as compared to pepsin, might be essential for the role of these enzymes in limited proteolytic processes. AMMERER et al. (2) and WOOLFORD et al. (32) independently demonstrated that mutant yeast strains deficient in proteinase yscA accumulate inactive precursors of proteinase yscB, carboxypeptidase yscY and other vacuolar hydrolases, suggesting a regulatory role of proteinase yscA for the activity of these enzymes. The subsite-specificity of proteinase yscA does, however, not directly account for the

observed dependence on proteinase yscA for hydrolase maturation in yeast (2, 19, 32). The cleavages necessary for "correct" N-terminal processing of proteinase yscB, carboxypeptidase yscY and proteinase yscA itself, occur between amino acids with low cleavage probability by proteinase yscA: Glu³-Ala²-Thr¹ - Glu¹-Phe²-Asp³ - in proteinase yscB (21), -Arg³-Val²-Asn¹ - Lys¹-Ile²-Lys³ - in carboxypeptidase yscY (30) and -Phe³-Thr²-Glu¹ - Gly¹-Gly²-His³ - in proteinase yscA itself (2, 32). Maturation of the precursor of proteinase yscB additionally involves sequential removal of C-terminal fragments, one of which is catalyzed by proteinase yscA (20). The exact position of the cleaved bond has not yet been established. Hydrolase maturation is multi-step limited proteolytic reactions, and proteinase yscA is most likely involved in cleavages leading to slightly higher molecular weight intermediates (19, 20). Such intermediates are catalytically active and might be physiologically important, although the proteinases obtained from cells at the end of growth and after extensive autoactivation of the cell lysate during enzyme purification have lower molecular weights.

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