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Biochemical characterization and structural prediction of a novel cytosolic leucyl aminopeptidase of the M17 family from *Schizosaccharomyces pombe*

Irma Herrera-Camacho[1], Nora H. Rosas-Murrieta[1], Arturo Rojo-Domínguez[2], Lourdes Millán[1], Julio Reyes-Leyva[3], Gerardo Santos-López[3] and Paz Suárez-Rendueles[4]

1 Laboratorio de Bioquímica y Biología Molecular, Centro de Química del Instituto de Ciencias, Universidad Autónoma de Puebla, Puebla, Mexico
2 Departamentos de Ciencias Naturales-Cuajimalpa y de Química-Iztapalapa, Universidad Autónoma Metropolitana, Mexico D. F., Mexico
3 Laboratorio de Virología y Biología Molecular, Centro de Investigación Biomédica de Oriente, IMSS, Atlixco-Metepec, Puebla, Mexico
4 Departamento de Bioquímica y Biología Molecular. Facultad de Medicina, Universidad de Oviedo, Spain

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Correspondence
I. Herrera-Camacho, Laboratorio de Bioquímica y Biología Molecular. Centro de Química del Instituto de Ciencias, Universidad Autónoma de Puebla, Apartado postal 1613, 72000 Puebla Pue, Mexico
Fax: +52 222 2295551
Tel: +52 222 2295500 ext.7295
E-mail: pherrera@siu.buap.mx

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A new leucyl aminopeptidase activity has been identified in the fission yeast *Schizosaccharomyces pombe*. The enzyme, which has been purified and named leucyl aminopeptidase yspII (LAP yspII), had a molecular mass of 320 and 54 kDa by gel filtration and SDS/PAGE, respectively, suggesting a homohexameric structure. The enzyme cleaved synthetic aminoacyl-4-nitroanilides at an optimum of pH 8.5, and preferred leucine and methionine as N-terminal amino acids. A clear dependence on Mn^{2+} concentration for activity was found, and an apparent association constant of 0.33 mM was calculated for the metal ion. Bestatin behaved as a competitive inhibitor of LAP yspII ($K_i = 0.14$ μM), while chelating agents such as chloroquine, EDTA and 1,10-phenanthroline also reduced enzyme activity. A MALDI-MS analysis, followed by sequencing of two of the resulting peptides, showed that LAP yspII undoubtedly corresponds to the putative aminopeptidase C13A11.05 identified in the *S. pombe* genome project. The protein exhibited nearly 40% sequence identity to fungal and mammalian aminopeptidases belonging to the M17 family of metallopeptidases. Catalytic residues (Lys292 and Arg366), as well as those involved in coordination with the cocatalytic metal ions (Lys280, Asp285, Asp303, Asp362 and Glu364) and those forming the hydrophobic pocket for substrate binding (Met300, Asn360, Ala363, Thr390, Leu391, Ala483 and Met486), were perfectly conserved among all known aminopeptidases. The *S. pombe* enzyme is predicted to be formed two clearly distinguished domains with a well conserved C-terminal catalytic domain showing a characteristic topology of eight β-sheets surrounded by α-helical segments in the form of a saddle.

Leucyl aminopeptidases (LAPs; EC 3.4.11.1) are members of the M17 family of metallo-peptidases [1] that cleave leucine residues from the N-terminus of proteins, peptides and synthetic substrates, although substantial activity may also be evident for other amino acids. They are widely distributed in organisms from bacteria to humans, the best characterized being those from *Bos taurus*, *Escherichia coli* and *Lycopersicon esculentum* (tomato) [2–4]. X-ray crystal structures have shown that these enzymes exist as homohexamers, comprising two trimers stacked on top of one another. In many cases, each monomer binds two zinc ions and folds into two α/β-type quasi-spherical globular domains, producing a comma-like shape [5],

Abbreviation
LAP, leucyl aminopeptidase; Lys-NA, lysine 4-nitroanilide.

although the *E. coli pepA*-encoded aminopeptidase presumably binds manganese and is inhibited by zinc through residues that are absolutely conserved and located in the C-terminal catalytic domain [3].

A wide collection of aminopeptidase activities have been described in the yeast *Saccharomyces cerevisiae*, the best characterized being vacuolar aminopeptidase I [6–8], cytosolic metallo-aminopeptidase II [9], bleomycin hydrolase (aminopeptidase yscIII) [10], aminopeptidase Y [11], aspartyl aminopeptidase and methionyl aminopeptidase, but not one has been classified as an M17 peptidase.

In the fission yeast *Schizosaccharomyces pombe*, whose genome sequence has been reported [12], there is limited information about peptidase activities. To date, only seven intracellular proteolytic enzymes have been described in this organism: two endoproteinases, named proteinases yspA and yspB [13], one carboxypeptidase yspI [14], one aminopeptidase yspI [15], one dipeptidyl aminopeptidase yspI [16], and two cytosolic proteolytic complexes, the multicorn peptidase [17] and proteasome 26S [18]. There is, however, a deduced amino acid sequence annotated as the putative aminopeptidase C13A11.05 that could belong to the M17 family of peptidases.

The biological roles of LAPs are not completely understood, and may be complex and species-specific. Interestingly, members of this group of enzymes are also implicated in transcriptional regulation, and are thought to combine catalytic and regulatory properties [19]. For example, the *E. coli* LAP serves as an aminopeptidase [20] and a DNA-binding protein that mediates both site-specific recombination and transcriptional control [3,19]. LAP from the basidiomycete *Coprinus cinereus* has been implicated in meiotic development [21]. On the other hand, the human LAP is induced by γ-interferon [22] and has been implicated in the processing of peptides released from the proteasome. These peptides are subsequently used for antigen presentation to the MHC I complex [23,24].

To understand the roles of LAPs in yeasts, it is critical to isolate and characterize an enzyme that may correspond to the putative aminopeptidase from the annotated genome sequence of the fission yeast *S. pombe* (C13A11.05). In this paper, we report on the biochemical and molecular characterization of a metalloaminopeptidase that was unequivocally identified as the translated product of the above-mentioned putative aminopeptidase gene. This new member of the M17 family was identified and purified in a *S. pombe* mutant strain lacking both aminopeptidase yspI and dipeptidyl aminopeptidase yspI. The enzyme was characterized as a hexameric manganese-dependent metallo-exopeptidase and named leucyl aminopeptidase yspII (LAP yspII). We also modelled the structure of the well-conserved C-terminal domain using bovine LAP as the enzyme model.

Results and Discussion

Identification of a new leucyl aminopeptidase activity in *S. pombe*

Aminopeptidases have been found in bacteria, yeast, plants, animal and human tissues [25]. In yeasts, a multitude of aminopeptidase activities were discovered by fractionation of *Sa. cerevisiae* crude extracts on ion-exchange and gel filtration resins [26], although only a few have been well characterized [6,7,9–11]. Their biological roles remain to be clearly established, probably due to the fact that overlapping substrate specificities make it almost impossible to identify any phenotype for the absence of a single enzyme [27]. The situation seemed to be much less complicated in the case of the fission yeast *S. pombe*, in which only one aminopeptidase activity, named aminopeptidase yspI and cleaving N-terminal lysine, had been biochemically and genetically characterized [15,28], although more recently a sequence (SPAC13A11.05) was reported by the fission yeast genome project that might encode a putative aminopeptidase.

Aminopeptidase yspI, the main aminopeptidase activity in the fission yeast, which shows a preference for basic residues, has been shown to be a dimer with an apparent molecular mass of 184 kDa that is composed of two identical subunits, inhibited by EDTA, chloroquine and mercury ions [15,28,29]. This aminopeptidase activity is easily detected as the major lysine 4-nitroanilide (Lys-NA) cleaving activity after gel filtration of a soluble extract of a *S. pombe* wild-type strain (Fig. 1A). To search for new aminopeptidase activities, we used several different aminoacyl-4-anilides as substrates, and performed activity tests at several pH values, with or without different metal ions as putative activators. Under these conditions, a new peak corresponding to an unidentified aminopeptidase-like activity was detected, cleaving L-leucine 4-nitroanilide (Leu-NA) at pH 8.5, activated by Mn^{2+}, and emerging from the column at an elution volume corresponding to 320 kDa (Fig. 1A). This new activity peak was easily detected in a mutant *S. pombe* strain $3Dh^{+}$ that is devoid of aminopeptidase yspI and dipeptidyl aminopeptidase yspI [16], thus confirming that it was not a residual activity of aminopeptidase yspI, but a different aminopeptidase activity (Fig. 1B), which we named leucyl aminopeptidase yspII (LAP yspII).

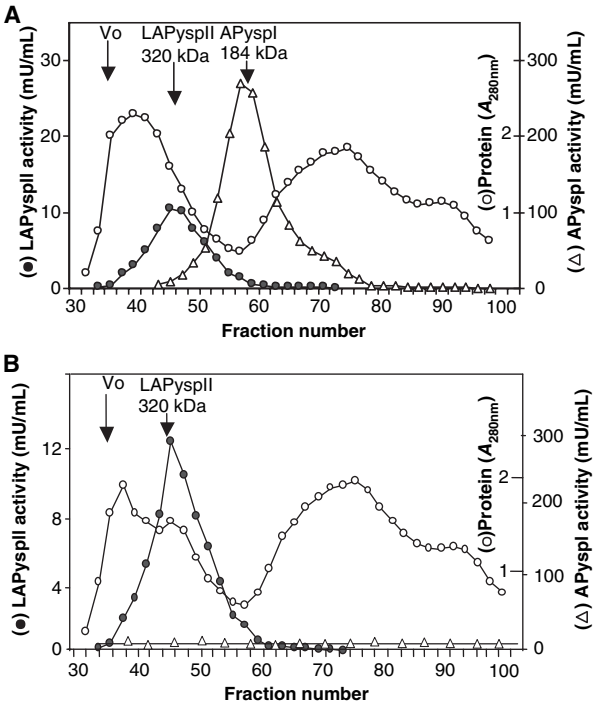

Fig. 1. Gel filtration of soluble extracts of *S. pombe*. Soluble extracts were prepared from a wild-type strain (A) or a mutant strain $3Dh^+$ devoid of aminopeptidase yspI and dipeptidyl aminopeptidase yspI (B), both grown in minimal medium at 30 °C and harvested near the end of the logarithmic phase of growth as described in Experimental procedures. Equal amounts of protein of each extract were applied to the column. Vo, elution volume of blue dextran.

Purification and biochemical characterization of LAP yspII

LAP yspII was purified from a crude extract prepared from *S. pombe* strain $3Dh^+$ that is devoid of both aminopeptidase yspI and dipeptidyl aminopeptidase yspI. The purification procedure is summarized in Table 1, and consisted of sequential gel-filtration chromatography, ion-exchange chromatography and affinity chromatography. A purification of about 250-fold, with a yield of 8.6%, was achieved.

Table 1. Purification of leucyl aminopeptidase yspII from *S. pombe*.

Purification step	Activity (mU)	Protein (mg)	Specific activity ($mU \cdot mg^{-1}$)	Yield (%)	Purification (fold)
Crude extract	477.3	610.3	0.78	100	1.0
Soluble extract	340.2	227.2	1.49	71.2	1.9
Sephacryl S-300HR	324.5	32.6	9.93	67.9	12.7
DEAE-cellulose	186.5	2.9	64.31	39.1	82.5
Leu-Phe–AH-Sepharose-4B	41.3	0.21	196.95	8.6	252.5

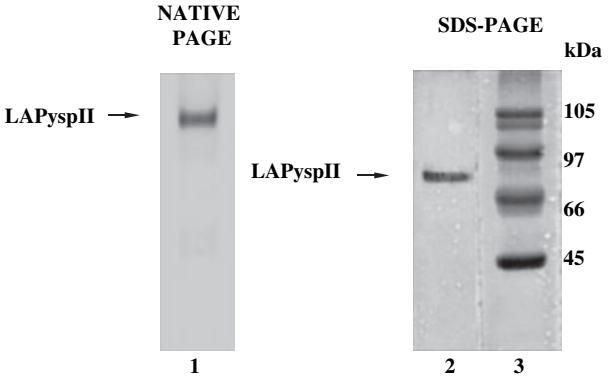

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) of purified leucyl aminopeptidase yspII. Lane 1, native PAGE of LAP yspII after the affinity chromatography step of the purification procedure. Lane 2, SDS/PAGE of the same type of sample. Lane 3, Molecular weight markers from Pharmacia. For details, see Experimental procedures.

A sample of the peak fraction of the affinity chromatography step was subjected to PAGE under native conditions, resulting in a single protein band (Fig. 2, lane 1), which, when cut out from the gel and incubated with the substrate Leu-NA and manganese, showed aminopeptidase activity (data not shown). Performing SDS/PAGE on the same type of sample resulted in a single band with a molecular mass of 54 kDa (Fig. 2, lane 2). The molecular masses of 320 kDa for the native enzyme (Fig. 1A) and 54 kDa for the denatured enzyme correlate well with a composition of six subunits per protein molecule. Thus native LAP yspII appears to be a homohexameric peptidase, as has been reported for other orthologous enzymes such as LAP from the fungus *C. cinereus* (Q8TGE4) and *B. taurus* (P00727).

Using Leu-NA as substrate, the optimum pH for the enzyme in Tris buffer was determined to be 8.5. When performing activity tests at this pH value, a clear dependence on Mn^{2+} concentration for activity was found, reaching maximal activation (five-fold) at a Mn^{2+} concentration of 0.5 mM or higher. We further investigated whether other divalent metal cations could replace Mn^{2+}, and found that Mg^{2+} and Ni^{2+} also enhanced aminopeptidase activity, while Ca^{2+}, Cu^{2+}, Ba^{2+} and Zn^{2+} had an inhibitory effect (data not shown).

A kinetic study of the saturation curves for the enzyme for the substrate Leu-NA performed using various Mn^{2+} concentrations (ranging from 0.2 to 2 mM) showed that V_{max} was not altered by the presence of Mn^{2+} ions, but the apparent K_m decreased as the

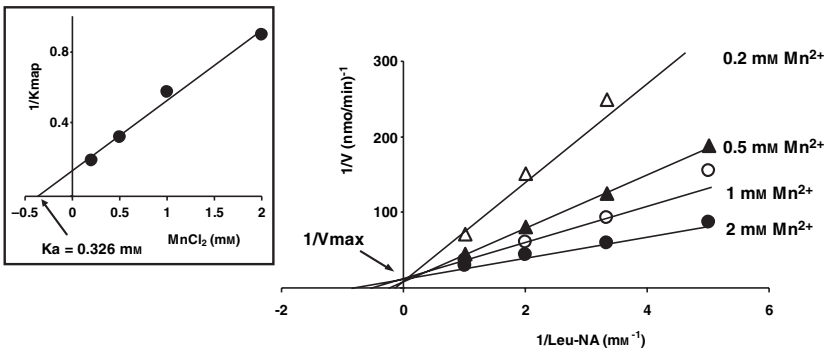

Fig. 3. Effect of Mn^{2+} ions on the kinetic parameters of purified leucyl aminopeptidase yspII. Hydrolysis of Leu-NA in 50 mM Tris-HCL buffer, pH 8.5, by purified LAP yspII (0.5 μg of protein in each test) was measured in the presence of various concentrations of Mn_2Cl. Data were plotted after Lineweaver–Burk transformation. Insert: determination of the apparent activation constant for Mn^{2+}.

Mn^{2+} concentration increased (Fig. 3). Thus, Mn^{2+} acts as a 'competitive activator' of the enzyme, the calculated apparent activation constant K_a being 0.326 mM (Fig. 3, insert), about 15-fold lower than the K_m for Leu-NA as substrate. This result clearly suggests that this metal is essential for catalysis, and thus the enzymatic reaction will not proceed unless an enzyme–metal complex is first formed to which the substrate can be bound, resulting in a ternary enzyme–metal–substrate complex that allows hydrolysis of a substrate peptide bond. Thus, LAP yspII should be considered as a metallo-enzyme, with Mn^{2+} ions involved in the catalytic mechanism.

The enzyme specificity of LAP yspII was investigated using a broad spectrum of various synthetic aminoacyl derivatives. The relative hydrolysis rates were estimated by a continuous assay, and the kinetic parameters were determined using Lineweaver–Burk plots. The only substrates cleaved are substrates of aminopeptidases. Neither the substrate Ala-Pro-NA (representing dipeptidyl aminopeptidases) nor the substrate *N*-benzoyl-L-tyrosyl-NA (representing carboxypeptidases) were cleaved by LAP yspII (Table 2). The enzyme showed a clear preference for leucine and methionine as N-terminal amino acids, but basic residues (Arg and Lys) were also cleaved but at a much reduced rate. This behaviour is typical of cytosolic leucyl aminopeptidases (EC 3.4.11.-). The K_m values calculated for the aminoacyl-4-nitroanilides corresponding to the four above-mentioned amino acids were very similar (ranging from 3.6 to 4.5 mM), and the differences in the relative rate of hydrolysis were due to differences in V_{max} (ranging from 280 nmol min^{-1} for Leu-NA to 102 nmol min^{-1} for Lys-NA) (Table 2).

From these substrate specificity studies, it seems that the nature of the amino acid side chain is important for substrate recognition (Leu, Met, Arg and Lys being bound with similar affinity, and all of them having an unsubstituted β-carbon), but differences were found in product formation and liberation (V_{max}). Substrates with a substituted β-carbon (Val) or lacking it (Gly) are not hydrolyzed. This geometrical selectivity might be mediated by the side chain of Lys292 whose relative position in the active site seems to depend on the nature of substrates and correlates with their relative activity. It should be kept in mind, however, that the kinetic tests were performed using artificial amino analogues (amino acyl-4-nitroanilides) as substrates, and these may be hydrolyzed at rates that are not comparable to the rates of hydrolysis of natural peptides that bear the same N-terminal residue.

The effect of various inhibitors on enzyme activity is summarized in Table 3. Bestatin, a specific inhibitor of metallo-enzymes [30], was a potent inhibitory agent of LAP yspII (50% residual activity at 0.1 μg mL^{-1} bestatin), confirming the metal-ion dependence of LAP yspII activity. The inhibition was shown to be of the competitive type, with a calculated K_i value of 0.14 μM

Table 2. Substrate specificity of leucyl aminopeptidase yspII. NA, 4-nitroanilide.

Substrate	Relative activity (%)[a]	K_m (mM)	V_{max} (nmol·min^{-1})
Leu-NA	100	4.5	280
Met-NA	88	4.4	220
Arg-NA	39	3.7	140
Lys-NA	30	3.6	102
Ala-NA	10		
Gly-NA	5		
Val-NA	4		
Phe-NA	4		
Pro-NA	2		
γ-Glu-NA	3		
Ala-Pro-NA	3		
N-benzoyl-L-tyrosyl-NA	0		

[a]Activity against all substrates was tested with purified enzyme and is expressed relative to the rate of hydrolysis of L-leucine-4-nitroanilide (100% was 240 mU mg^{-1}). Activities were measured at pH 8.5 with 4 mM substrate, 1 mM $MnCl_2$ at 37 °C.

Table 3. Effect of protease inhibitors on leucyl aminopeptidase yspII activity.

Inhibitors	Final concentration	Residual activity (%)[a]
None	–	100[a]
Bestatin	0.1 μg mL^{-1}	51
Antipain	10 μg mL^{-1}	98
Leupeptin	10 μg mL^{-1}	88
1,10-Phenanthroline	1 mM	62
EDTA	1 mM	44
Chloroquine	1 mM	30
Phenylmethanesulfonyl fluoride	5 mM	92
Tosyl-lysine chloromethyl ketone (TLCK)	5 mM	81

[a]Purified leucyl aminopeptidase yspII (210.30 mU mg^{-1}) was preincubated in the presence of the indicated inhibitors at 37 °C for 15 min in 50 mM Tris/HCl, pH 8.5, 1 mM $MnCl_2$. Thereafter, enzyme activity was determined using Leu-NA as substrate as described in Experimental procedures.

(data not shown). Chelating agents such as 1,10-phenanthroline, EDTA and chloroquine reduced LAP yspII activity by 28–70% at concentrations of 1.0 mM. Antipain and leupeptin, inhibitors of serine and cysteine peptidases, respectively, had no effect on enzyme activity even at concentrations of 10 μg mL^{-1}. The same applies to phenylmethanesulfonyl fluoride and tosyl-lysine chloromethyl ketone (both 5 mM), which are also inhibitors of serine proteases.

Identification of a putative aminopeptidase annotated in the *S. pombe* genome project as LAP yspII

The stained protein band corresponding to SDS/PAGE electrophoresis of a purified sample of LAP yspII (equivalent to that of lane 2 in Fig. 2) was cut from the gel, dehydrated and sent to the Beckman Centre, Stanford University Medical Centre (Stanford, CA, USA), for MALDI-MS analysis. The masses of 66 tryptic peptides were measured and compared with those of known proteins contained in the data bank, and it was determined that the theoretical peptide mass of 60% of the sequence of the putative aminopeptidase C13A11.05 identified by the *S. pombe* genome project (http://www.sanger.ac.uk/Projects/S_pombe) matched that found in 32 of the tryptic peptides. To further confirm this finding, two of the tryptic peptides from LAP yspII were purified by HPLC and sequenced in Beckman Center. A BLAST analysis of the sequences showed that they were indeed contained in the deduced sequence of the putative aminopeptidase mapping to chromosome I of *S. pombe*. Sequence analysis of this region uncovered an ORF of 1539 nucleotides coding for a protein of 513 amino acids (Fig. 4), with a predicted molecular mass of 56.2 kDa, matching almost perfectly the value of 54 kDa determined for the monomer of LAP yspII by SDS/PAGE. We suggest naming the corresponding gene *ape2*.

A search for regulatory regions within the 5′ UTR gave the following results. A putative TATA box, TATAATAA, was located 108 bp upstream of the predicted ATG codon (Fig. 4). Three GAAT consensus sequences for binding of the transcriptional activator GCN4 [31] were found at positions −131, −172 and −290. Also, three GATA/CTAT consensus sequences for binding of GATA factors [32,33], frequently found in nitrogen catabolite repression genes, were found at positions −31, −510 and −580 (Fig. 4), suggesting possible transcriptional regulation of LAP yspII expression by nutrient limitation, as has been shown to be the case with several *Sa. cerevisiae* peptidases [8]. Even though introns are frequent in *S. pombe* genes [34], we could not find any consensus sequence for the presence of introns among the 2340 bp examined.

Inspection of the deduced amino acid sequence of *S. pombe ape2* revealed the absence of a signal peptide consensus sequence, long hydrophobic domains or signals for retention in the endoplasmic reticulum, nucleus or vacuole. The most probable site for subcellular localization of the predicted protein is the cytosol, as deduced from the presence of the NTDAEGRL motif at position 360 (Fig. 4).

Comparison of the predicted amino acid sequence with protein sequences in the EMBL and Swiss Prot databases revealed 37% identity with LAP from the fungus *C. cinereus* (Q8TGE4), 35% identity with the 487-amino acid sequence of *B. taurus* LAP (P00727), 35% identity with human leucyl aminopeptidase (P228838), 36% identity with mouse LAP3 (Q9CPY7), and 28% identity with *E. coli* cytosol aminopeptidase (P68767). The sequence similarity was even higher (47, 49, 48, 49 and 35%, respectively) when C-terminal domains of the different aminopeptidases, containing their respective active sites, were considered. All the residues that appear to be involved in metal-ion binding, catalysis or substrate binding are conserved in all those aminopeptidases (Fig. 5), except for one hydrophobic residue (Val452 in the yeast enzyme) that showed conservative changes in the other species.

The Pfam database was used to identify a protein family to which LAP yspII could be assigned, and it was found that the protein sequence belongs to

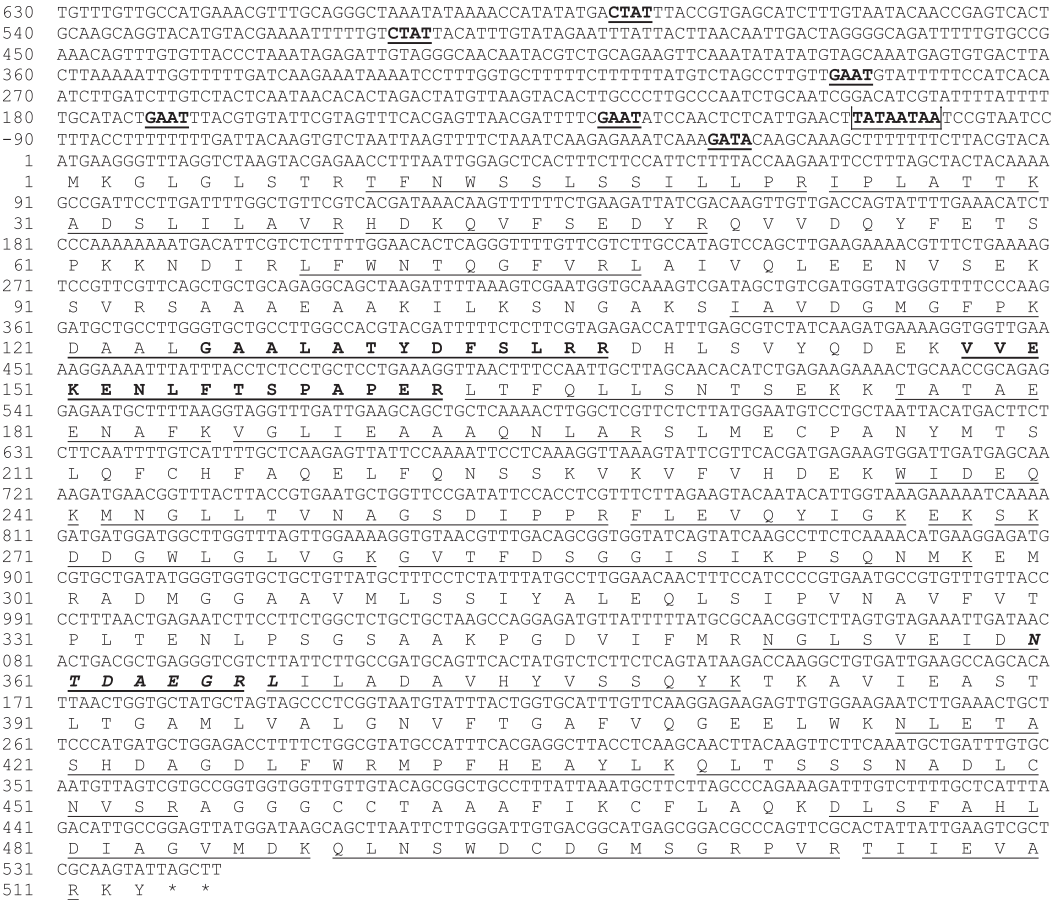

Fig. 4. Nucleotide sequence of the annotated genome sequence of *S. pombe* SPC13A11.05, and deduced amino acid sequence of leucyl aminopeptidase yspII. Underlined residues, tryptic peptides generated after MALDI-MS analysis of an SDS/PAGE sample of purified LAP yspII. Underlined residues: tryptic peptides generated after MALDI-MS analysis of purified LAP yspII. Underlined bold residues: peptides from LAP yspII that were HPLC purified and sequenced (see text for details). Bold residues in italics: motif present in cytoplasmic proteins. In the 5′ UTR, boxed bold TATAATAA: a TATA-like sequence and the bold underlined bases: putative regulatory sequences.

peptidase M17 family, for which the metallopeptidase leucyl aminopeptidase from *B. taurus* was the first described and is considered the representative enzyme [5]. Its tertiary structure has been resolved, revealing a two-domain structure, with the active site in the most conserved C-terminal domain, covering residues from 194–508 in the case of LAP yspII, while the N-terminal domain of the yeast enzyme (much more variable among members of the family) extends from residues 35–139.

All the aminopeptidases included in the M17 family of peptidases possess two metal ions that are essential for catalysis [35]. The metal ions are both zinc in mammalian aminopeptidases [36], but are manganese in bacterial aminopeptidase A [3]. The *S. pombe* LAP yspII display a clear dependence on Mn^{2+} ions for maximal activity, while Zn^{2+} inhibits enzyme activity; thus, in this respect, LAP yspII resembles the bacterial aminopeptidase.

Members of the M17 family of peptidases have been shown to consist of two unrelated functional domains: a unique N-terminal domain, which might play a regulatory function (covering residues 35–139 in the case of LAP yspII), and a well-conserved catalytic C-terminal domain (extending from residues 194–508 in the case of LAP yspII) (see Fig. 5). In a multiple sequence alignment of LAP yspII with leucyl aminopeptidases of the M17 family from fungus, mammals and bacteria, the main differences were found in the sequence covering the N-terminal domain of the *S. pombe* enzyme (two 15-residue insertions, one 12-residue insertion, and a 11-residue deletion with respect to bovine LAP; data not shown), while minor differences (one 2-residue insertion, two 1-residue insertions, and three

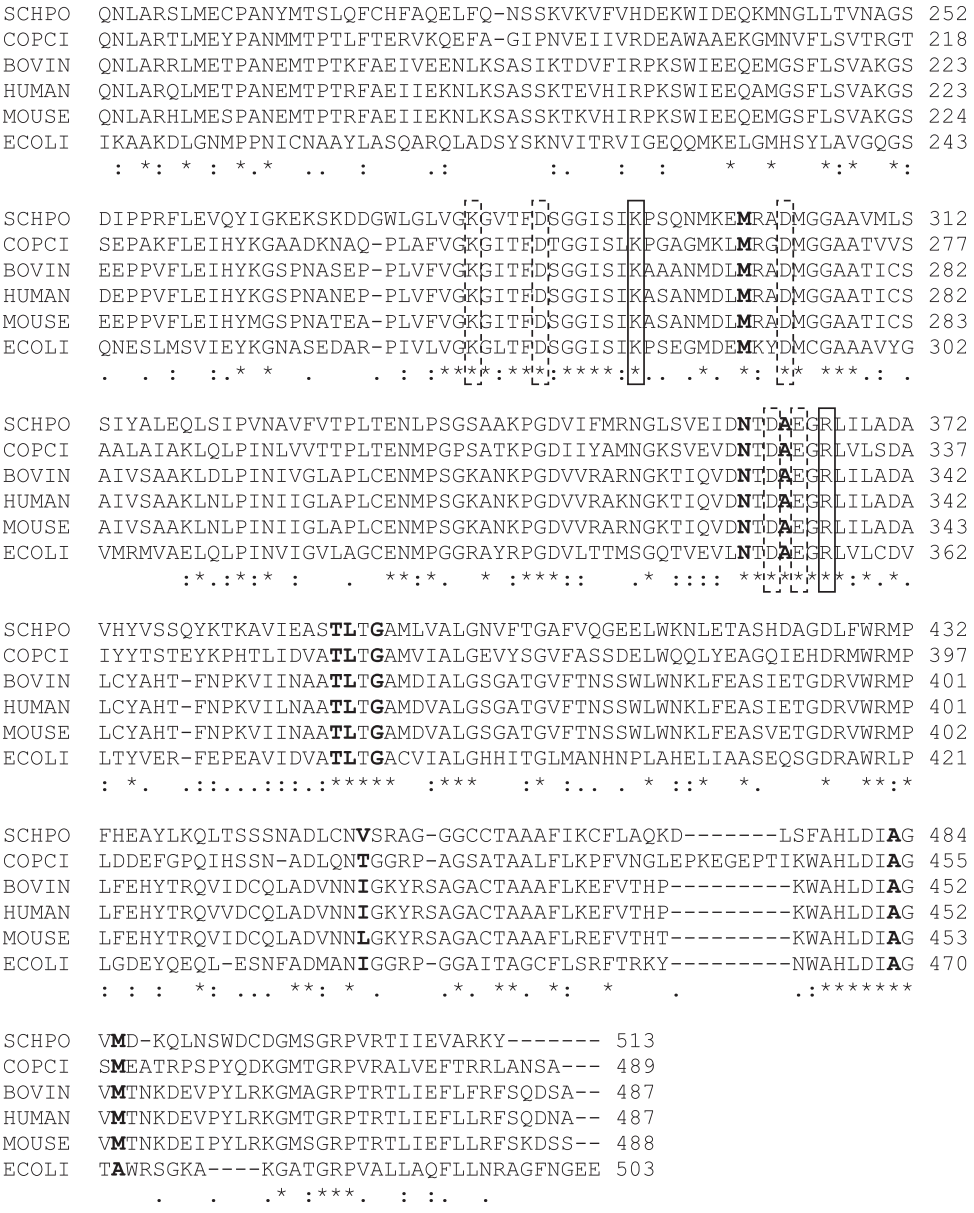

Fig. 5. Alignment of the C-terminal domain sequences of leucyl aminopeptidase yspII (SCHPO), and leucine aminopeptidases from *C. cinereus* (COPCI), *B. taurus* (BOVIN), human (HUMAN), mouse (MOUSE) and *Escherichia coli* (ECOLI).Solid boxes, putative catalytic residues. Dashed boxes, residues involved in coordination with the co-catalytic metal ion. Bold residues, amino acids forming the hydrophobic pocket that defines substrate specificity.

1-residue deletions) were found when the C-terminal domains of the fission yeast and bovine LAP were compared (see Fig. 5 for the C-terminal alignment).

The high conservation of the primary structure when the C-terminal domains of the above-mentioned M17 peptidases were compared allowed us to confirm the identity of the previously predicted active residues (Lys292 and Arg366), as well as those involved in the coordination with the co-catalytic metal ions (Lys280, Asp285, Asp303, Asp362 and Glu364) of the yeast enzyme. The hydrophobic pocket that defines the substrate specificity of this type of aminopeptidases was also highly conserved in the yeast enzyme, and was formed by residues Met300, Asn360, Ala363, Thr390, Leu391, Gly393, Ala483 and Met486. Only one residue (Val452) was different between the aligned sequences, but this corresponded to conservative changes (see Fig. 5).

Prediction of the secondary and tertiary structure of LAP yspII

Because of the high degree of similarity shared by LAP yspII and LAP from *B. taurus*, a perfectly characterized enzyme, we were able to obtain some insight into the structure of the yeast enzyme taking the mammalian enzyme as a model. We used the deduced amino acid sequence of LAP yspII and the GOR IV method [37] to predict its secondary structure, and obtained a very clear pattern of alternating α-helical segments and β-sheets all along the sequence, confirming that the yeast enzyme is a protein of the α/β type [38], as has been shown to be the case with the cattle enzyme. The secondary structure content of the yeast enzyme calculated by GOR IV was the following: 45.03% α-helix, 14.04% β-sheet and 40.94% aleatory coil. We also used the JPRED method [39], which gave a content of 29.8% α-helix, 15.59% β-sheet and 54.58% aleatory coil for the deduced amino acid sequence of the *ape2* gene. The apparent discrepancies found with the different methods may be explained by taking into account that the secondary structure predicted was only reliable when the C-terminal segment (amino acids 194–508) was considered, as the similarity between LAP yspII and those used for comparison was low for the N-terminal domain (amino acids 35–139).

Knowledge of the three-dimensional structure of a protein is essential to gain insight into its cellular function. The difficulties encountered in obtaining crystals with a three-dimensional net that was ordered and stable enough to be studied led to the use of various algorithms and computer packages to predict the structure of a protein from its amino acid sequence, which is possible provided the three-dimensional structure of a homologous protein with sequence similarity has been previously resolved [40]. Taking advantage of the fact that LAP yspII and bovine LAP shared 35% sequence identity, which increased up to 49% when comparing their respective C-terminal domains (Fig. 5), and that the structure of the monomer, trimer and hexamer of the LAP from *B. taurus* has been resolved by X-ray crystallography [36,41], we undertook the construction of a three-dimensional model of the whole amino acid sequence of the yeast enzyme, which was aligned with bovine LAP for modeling using the MOE program. The result obtained showed that *S. pombe* LAP yspII is composed two clearly distinguished domains (Fig. 6A): a short N-terminal domain, encompassing residues 35–139, that is highly variable in the folding, and a longer and much more conserved C-terminal domain extending from amino acids 194–508. The *B. taurus* LAP monomer structure is shown in Fig. 6(B). We therefore used various programs to model the isolated C-terminal domain of the yeast enzyme, using crystals of bovine LAP that were either free or complexed with several inhibitors as protein models [5,41,42]. The result (see Fig. 6C) showed a C-terminal domain folding of the yeast enzyme similar to that reported for the cattle enzyme, consisting of alternate α-helix and β-sheet segments, with a characteristic topology of eight β-sheets surrounded by α-helical segments in the form of a saddle, and containing a full catalytic site.

The C-terminal domain of bovine LAP accommodates its active site, made up of two catalytic residues (Lys262 and Arg336) and five residues (Lys250, Asp255, Asp273, Asp332 and Glu334) co-ordinating the two Zn ions. Zn1, located in the so-called structural site is essential for biological activity of the enzyme and is bound by the carboxylates of Asp255, Asp332 and Glu334. Zn2, which is located in the

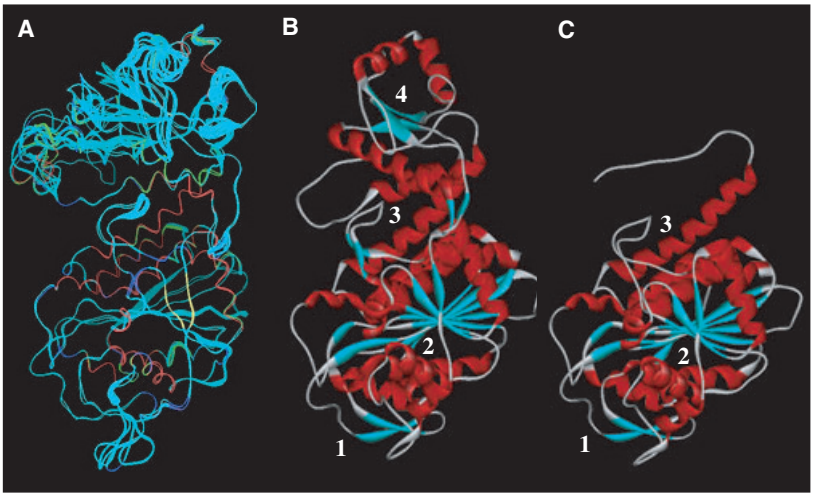

Fig. 6. Molecular models of leucyl aminopeptidases. (A) N-terminal (up) and C-terminal (down) domains of leucyl aminopeptidase yspII generated with the aid of the MOE program. (B) N-terminal (up) and C-terminal (down) domains of leucyl aminopeptidase from *B. taurus* by X-ray crystallography [36,41]. (C) Model of C-terminal domain of leucyl aminopeptidase yspII generated with SWISS MODEL. 1, small β-chains on the monomer surface. 2, bundle of β-sheets surrounded by α-helix segments with the form of a saddle. 3, α-helix segments surrounding the β-sheets in a sandwich-like form. 4, N-terminal domain of *B. taurus*-LAP.

activation or regulation site, may be interchanged with other divalent cations such as Mg^{2+}, Mn^{2+} or Co^{2+}, and is bound by the amino group of Lys250 and the carboxylates of Asp273 and Glu334. Glu334 binds both metal ions, and the backbone carbonyl group of Asp332 coordinates both metals. A single hydroxyl group originating from a polarized water molecule bridges both metal ions and acts as a nucleophil on the substrate molecule during catalysis. Lys262 is important for stabilization of the substrate–enzyme complex, which is known as a gem diolate [36,41,43]. All these residues were found to be conserved in LAP yspII in the multiple sequence alignments performed (Fig. 5), such that Lys292 and Arg366 are predicted to be two catalytic residues, and Lys280, Asp285, Asp303, Asp362 and Glu364 are predicted to be the five amino acid metal ligands in the fission yeast enzyme, in which metal site 1 may be supposed to be occupied by a Zn^{2+} ion, whereas in regulatory site 2 this metal ion may be replaced by Mn^{2+}, a potent activator of LAP yspII. It is worth noting that two of the residues bound to metal site 1 (Asp362 and Glu364) are separated only by one residue (Ala363), while the third residue (Asp285) is separated by 77 residues from Asp362, which is a spacing sequence that confers the optimal structural characteristics to bring about catalysis.

Access of substrates to the active centre of some peptidases is determined by the presence of a hydrophobic pocket, which is located near the surface of the monomer in bovine LAP and whose residues were found to be conserved in the case of the yeast enzyme, except for Val452 which corresponded to Ile421 of bovine LAP (Fig. 5). Thus, the hydrophobic pocket of yeast LAP yspII is predicted to be formed of Met300, Asn360, Ala363, Thr390, Leu391, Gly393, Val452 and Met486, whereby the pocket wall is built up by Asn, Val, Leu and Gly, while Met300 defines the maximal length of the polypeptide chain of the substrate.

Taking all these data into account, we propose that the most probable composition and three-dimensional disposition of the active centre of LAP yspII is that shown in Fig. 7, which was generated using SWISS MODEL (Fig. 7A) and CHEMDRAW PRO (Fig. 7B). According to the criteria described by Vallee [44,45], we consider that Asp285 and Asp362, defining the spacing sequence, are the basis for the structural organization of the active centre, whose function is determined by the catalytic residues located either inside (Lys262) or near the linking residues but outside of the spacing sequence (Arg366).

Taking together all the evidence presented here, we conclude that LAP yspII is a homohexameric metalloaminopeptidase belonging to the M17 peptidase family. This family is absent in baker's yeast (*Sa. cerevisiae*), and therefore this is one of only four members of the M17 peptidase family identified in fungi. In addition, the *Schizosaccharomyces* enzyme is activated by manganese ions, and thus differs considerably from the cattle leucyl aminopeptidase in terms of folding of the N-terminal domain, and should be considered a novel enzyme.

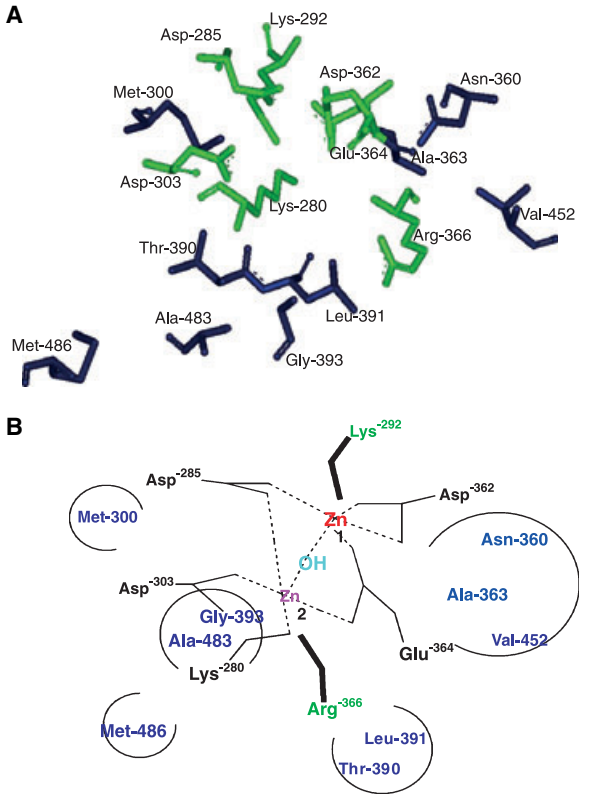

Fig. 7. Disposition of the active centre of LAP yspII containing two Zn atoms and one hydroxyl group. The catalytic residues are shown in green. Residues forming the hydrophobic cavity of substrate recognition are shown in blue. (A) Model generated using the WEB LAB VIEWER program. (B) Model generated using the CHEM DRAW PRO program.

Experimental procedures

Chemicals

All chromogenic peptide substrates, as well as the proteinase inhibitors used, were purchased from either Sigma (St Louis, MO, USA) (Leu-NA, bestatin, antipain, leupeptin, 1,10-phenanthroline, EDTA, chloroquine, phenylmethanesulfonyl fluoride, TLCK) or Bachem (Bubendorf, Switzerland) (Leu-Phe, Met-NA, Arg-NA, Lys-NA, Ala-NA, Gly-NA, Val-NA, Phe-NA, Pro-NA, c-Glu-NA, Ala-Pro-NA, *N*-benzoyl-L-tyrosyl-NA). Chromatographic media were obtained

from Pharmacia Biotech (Uppsala, Sweden) (AH-sepharose 4B and DEAE-cellulose) and Sigma-Aldrich (sephacryl S-300-HR). Yeast growth media (YNB) were obtained from Difco (Sparks, MD, USA). All other chemicals were from the highest purity available and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich.

Yeast strains and growth conditions

S. pombe mutant strain $3Dh^+$ (leu1-32, ura4-D18, ade6-250, ape1$^-$, dpa1$^-$), devoid of aminopeptidase yspI and dipeptidyl aminopeptidase yspI [16], was used for identification and purification of LAP yspII. The haploid wild-type strain 972 h^- was used as a control.

Yeast cells were grown in minimal medium containing 0.7% yeast nitrogen base without amino acids, with 2% glucose, 1.0 g L^{-1} sodium acetate, 0.9 g L^{-1} potassium chloride, 250 mg L^{-1} leucine, 75 mg L^{-1} uracil and adenine, and supplemented with a vitamin complex of 1 mg L^{-1} citric acid, 100 mg L^{-1} nicotinic acid and 100 mg L^{-1} *m*-inositol. Strains were incubated in a rotatory shaker at 30 °C and harvested near the end of the logarithmic growth phase ($A_{600\ nm} = 3.0$).

Enzyme assays

LAP yspII activity was measured against chromogenic peptide substrates. Leucyl aminopeptidase activity was routinely assayed at 37 °C using L-leucine-4-nitroanilide (Leu-NA) as substrate. The enzyme (100 μL) was preincubated with 1.0 mM $MnCl_2$ in 50 mM Tris/HCl buffer, pH 8.5, for 10 min. Thereafter, the substrate (1.0 mM Leu-NA) was added and the mixture further incubated at 37 °C. Reaction was stopped by adding 0.5 mL of 40 mM EDTA (pH 8.0) and 20 mM chloroquine. After centrifugation at 10 000 ***g*** for 10 min at 4 °C, liberation of 4-nitroanilide ($\lambda = 405$ nm) in the clear supernatant was measured in a UV-160 Shimadzu (Columbia, MD, USA) spectrophotometer. One enzyme activity unit is defined as the amount of enzyme that catalyses the release of 1 μmol of product min^{-1} under the test conditions. Enzyme activity was calculated using a molar absorption coefficient $\varepsilon_{405\ nm}$ of 9900 M^{-1} cm^{-1} for 4-nitroanilide.

Protein determination

Protein was determined using the method described by Sedmak and Grossberg [46] with crystalline BSA as standard.

Purification of the enzyme

All purification and chromatographic steps were carried out at 4 °C. Purification was followed by testing all fractions for aminopeptidase activity using Leu-NA as substrate.

Step 1. Preparation of soluble extract

A 22 g sample of cells (wet weight) grown up to the end of the logarithmic phase in minimal medium was harvested and disintegrated as described by Arbesú et al. [15]. The homogenate was centrifuged for 60 min at 100 000 × g.

Step 2. Gel-filtration chromatography

The soluble extract was applied to a Sephacryl S-300-HR column (2.6 × 50 cm, 250 mL of gel) previously equilibrated with 20 mM Tris/HCl buffer (pH 8.5) containing 100 mM KCl and 0.03% of Na_3N. The flow rate was 40 mL h^{-1}. Fractions of 1 mL were collected and tested for aminopeptidase activity. Those fractions showing the highest activity were pooled.

Step 3. Ion-exchange chromatography

The pooled enzyme fractions from step 2 were dialyzed against 20 mM Tris/HCl buffer (pH 8.5) and then loaded onto a DEAE-cellulose anion exchange column (1.5 × 20 cm) previously equilibrated with the same buffer. Protein was eluted from the column using an increasing salt gradient (100 mL, from 0.04–0.08 M KCl) at a flow rate of 15 mL h^{-1}, and collected in 2 mL fractions. The enzyme eluted from the column at a salt concentration of 0.06 M.

Step 4. Affinity chromatography

The affinity gel matrix was prepared by coupling the Leu-Phe dipeptide to AH-Sepharose-4B using the carbodiimide method recommended by Pharmacia-Biotech. The proteins of the DEAE-cellulose pool (12 mL, 0.80 mg of protein) were applied to a Leu-Phe–AH-Sepharose-4B column (1.5 × 10 cm, 18 mL of gel) previously equilibrated with 20 mM Tris/HCl buffer (pH 8.5). Proteins were eluted at a flow rate of 12 mL h^{-1} with a step salt gradient (15 mL of 0.1 M KCl, 20 mL of 0.15 M KCl, 15 mL of 0.7 M KCl). The enzyme eluted from the column at a salt concentration of 0.15 M. Fractions showing the highest activity were pooled, dialyzed against Tris/HCl buffer (pH 8.5), and stored at 4 °C for further use.

Native molecular mass determination

The soluble extract (from purification step 1) or molecular mass standards were applied to the Sephacryl S-300-HR column and eluted under the conditions described in purification step 2. Standard proteins (Pharmacia) were: egg albumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle aldolase (160 kDa), bovine liver catalase (232 kDa) and horse spleen ferritin (450 kDa).

Electrophoresis

Purified LAP yspII (5 μg of protein) was subjected to SDS/PAGE in 12% gels [47]. The gels were stained with 0.04% Coomassie G-250. Molecular weight marker proteins (Pharmacia) were used as standards. Nondenaturing polyacrylamide slab gels with discontinuous pH were prepared as described previously [48]. The stacking gel was 3.5% and the separating gel was 6%.

Determination of pH optimum and manganese dependence

An aliquot of 0.1 milliunits of purified LAP yspII (0.5 μg of protein) was included in the tests using Leu-NA as substrate. Buffers were adjusted with HCl to pH values ranging from 6.0 to 9.0.

Inhibition studies

Samples with 0.1 milliunits of purified LAP yspII, in 100 μL of 50 mM Tris/HCl buffer (pH 8.5) and 1.0 mM $MnCl_2$, were preincubated for 15 min at 37 °C with various concentrations of the putative inhibitors. Enzyme tests were started by the addition of 50 μL of 10 mM Leu-NA.

Kinetic studies

All kinetic studies were carried out at 37 °C using a D-160 Shimadzu spectrophotometer, employing a TCC-controller Shimadzu Peltier system to keep temperature constant. Reaction rates were determined in a continuous assay, and the release of 4-nitroaniline was monitored by the change in absorbance at 405 nm. The kinetic parameters were determined using Lineweaver–Burk plots and a program for regression and variance analysis.

MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) analysis

MALDI-MS analysis of a sample of purified LAP yspII subjected to SDS/PAGE and stained with 0.1% Coomassie R-250 was performed in the Beckman Centre, Stanford University Medical Centre (Stanford, CA, USA).

Bioinformatic analysis

The ORF corresponding to the sequence obtained by MALDI-MS analysis was searched by BLAST using alternative yeast nuclear genetic code and molecular weight calculation. Multiple alignments of deduced amino acid and nucleotide sequences were performed with CLUSTALW (http://www.ebi.ac.uk/clustalw/index.html). Prediction of regulatory regions was searched by means of MATINSPECTOR version 2.2 (http://www.gsf.de/biodv/matinspector.html). Homology modelling of C-terminal domain of yeast aminopeptidase yspII was partially performed using SWISS MODEL (http://swissmodel.expasy.org/) and CPH (http://www.cbs.dtu.dk/services/CPHmodels/) [49] programs, and further processed using MOE (http://www.chemcomp.com).

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References

1 Matsui M, Fowler JH & Walling LL (2006) Leucine aminopeptidases: diversity in structure and function. *Biol Chem* **387**, 1535–1544.
2 Kim H & Lipscomb WN (1994) Structure and mechanism of bovine lens leucine aminopeptidase. *Adv Enzymol Relat Areas Mol Biol* **68**, 153–213.
3 Sträter N, Sherrat DJ & Collons SD (1999) X-ray structure of aminopeptidase A from *Escherichia coli* and a model for the nucleoprotein complex in Xer-site specific recombination. *EMBO J* **18**, 4513–4522.
4 Chao-Jung T, Sang-Youl P & Walling L (2003) Isolation and characterization of the neutral leucine aminopeptidase (LapN) of tomato. *Plant Physiol* **132** (1), 243–255.
5 Burley SK, David PR, Taylor A & Lipscomb W (1990) Molecular structure of leucine aminopeptidase at 2.7 Å resolution. *Proc Natl Acad Sci USA* **87**, 6878–6882.
6 Cuevas R, Garcia-Alvarez N & Suárez-Rendueles P (1989) Yeast vacuolar aminopeptidases yscI. Isolation and regulation of the APE1 (LAP4) structural gene. *FEBS Lett* **259**, 125–129.
7 Klionsky DJ, Cuevas R & Yaver DS (1992) Aminopeptidase I of *Saccharomyces cerevisae* is localized to the vacuole independent of the secretory pathway. *J Cell Biol* **119**, 287–299.
8 Bordallo J & Suárez-Rendueles P (1995) Identification of regulatory proteins that might be involved in carbon catabolite repression of the aminopeptidase I gene of the yeast *Saccharomyces cerevisae*. *FEBS Lett* **376**, 120–124.
9 Garcia-Alvarez N, Cuevas R & Suarez-Rendueles P (1991) Molecular cloning of soluble aminopeptidase from *Saccharomyces cerevisae*. Sequence analysis of aminopeptidase yscII, a putative zinc-metallopeptidase. *Eur J Biochem* **202**, 993–1002.

10 Enenkel C & Wolf D (1993) BLH1 codes for a yeast thiol aminopeptidase, the equivalent of mammalian bleomycin hydrolase. *J Biol Chem* **268**, 7036–7043.
11 Herrera-Camacho I, Morales-Monterrosas R & Quiróz-Alvarez R (2000) Aminopeptidase yscCo-II: a new cobalt-dependent aminopeptidase from yeast – purification and biochemical characterization. *Yeast* **16**, 219–229.
12 Wood V, Volckaert G, Barrell BG & Nurse P (2002) The genome sequence of *Schizosaccharmyces pombe*. *Nature* **415**, 871–880.
13 Suárez-Rendueles P, Villa L, Arbesú MJ & Escudero B (1991) The proteolytic system of the fission yeast *S. pombe*. *FEMS Microbiol Lett* **81**, 215–220.
14 Tabuchi M, Iwaihura O, Ohtani Y, Ohuchi N, Sakurai J, Morita T, Iwahara S & Takegawa K (1997) Vacuolar protein sorting in fission yeast: cloning biosynthesis, transport and processing of carboxipeptidase Y from *Schizosaccharomyces pombe*. *J Bacteriol* **179**, 4179–4189.
15 Arbesú MJ, Gascon S & Suárez-Rendueles P (1991) Isolation and characterization of *S. pombe* mutants lacking aminopeptidase activity. *Yeast* **7**, 525–531.
16 Villa L & Suárez-Rendueles P (1994) Dipeptidil aminoeptidase yspI mutants of *S. pombe*: genetic mapping of *dpa1*$^+$ on chromosome III. *FEMS Microbial Lett* **120**, 211–216.
17 Osmulski PA & Gaczynska M (1998) A new large proteolytic complex distinct from the proteasome is present in the cytosol of fission yeast. *Curr Biol* **8**, 1023–1026.
18 Wilkinson CR, Penney M, McGurk G, Wallace M & Gordon C (1999) The 26S proteasome of the fission yeast *S. pombe*. *Philos Trans R Soc London [Biol]* **354**, 1523–1532.
19 Charlier D, Kholti A, Huysveld N, Gigot D, Maes D, Thia-toong TL & Glansdorff N (2000) Mutational analysis of *Escherichia coli* PepA, a multifunctional DNA-binding aminopeptidase. *J Mol Biol* **302**, 411–426.
20 Vogt VM (1970) Purification and properties of an aminopeptidase from *Escherichia coli*. *J Biol Chem* **245**, 4760–4769.
21 Ishizaki T, Tosaka A, Nara T, Aoshima N, Namekawa S, Watanabe K, Hamada F, Omori A & Sakaguchi K (2002) Leucine aminopeptidase during meiotic development. *Eur J Biochem* **269**, 826–832.
22 Harris CA, Hunte B, Krauss MR, Taylor A & Epstein LB (1992) Induction of leucine aminopeptidase by interferon-γ. *J Biol Chem* **267**, 6865–6869.
23 Beninga J, Rock KL & Goldberg AL (1998) Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J Biol Chem* **273**, 18534–18542.
24 Hattori A & Tsujimoto M (2004) Processing of antigenic peptides by aminopeptidases. *Biol Pharm Bull* **6**, 777–780.
25 Taylor A (1993) Aminopeptidases: towards a mechanism of action. *Trends Biochem Sci* **18**, 167–171.
26 Achstetter T, Ehmann C & Wolf DH (1983) Proteolysis in eukaryotic cells: aminopeptidases and dipeptidyl aminopeptidases of yeast revisited. *Arch Biochem Biophys* **226**, 292–305.
27 Suárez-Rendueles P & Wolf DH (1988) Proteinase function in yeast: biochemical and genetic approaches to a central mechanism of post-translational control in the eukaryote cell. *FEMS Microbiol Rev* **4**, 17–45.
28 Arbesú MJ, Valle E & Suáres-Rendueles P (1993) Purification and characterization of aminopeptidase yspI from *S. pombe*. *Yeast* **9**, 637–644.
29 Atmanspacher D & Rhön KH (1981) Only one aminopeptidase in *Schizosaccharomyces pombe*. *Z Physiol Chem* **362**, 459–463.
30 Umezawa H, Aoyagi T, Suda H, Hamad M & Taheuchi T (1976) Bestatin an inhibitor of aminopeptidase B, produced for *Actinomyces*. *J Antibiot* **29**, 97–99.
31 Grundmann O, Mosch HU & Braus GH (2001) Repression of GCN4 mRNA translation by nitrogen starvation in *Saccharomyces cerevisiae*. *J Biol Chem* **276**, 25661–25671.
32 Kuruvilla FG, Shamji AF & Schreiber SL (2001) Carbon- and nitrogen-quality signaling to translation are mediated by distinct GATA-type transcription factors. *Proc Natl Acad Sci USA* **98**, 7283–7288.
33 Tao Y & Marzluf GA (1999) The NIT2 nitrogen regulatory protein of Neurospora: expression and stability of nit-2 mRNA and protein. *Curr Genet* **36**, 153–158.
34 Kupfer DM, Drabenstot SD, Buchanan KL, Lai H, Zhu H, Dyer DW, Roe BA & Murphy JW (2004) Introns and splicing elements of five diverse fungi. *Eukaryotic Cell* **3**, 1088–1100.
35 Rawlings ND & Barrett AJ (1995) Evolutionary families of metallopeptidases. *Methods Enzymol* **248**, 183–228.
36 Sträter N & Lipscomb WN (1995) Two metal ion mechanism of bovine lens leucine aminopeptidase: active site, solvent structure and binding mode of ι-leucinal, a gem-biolato transition state analogue by X-ray crystallography. *Biochem* **34**, 14792–14800.
37 Garnier J, Gibrat JF & Robson B (1996) GOR secondary structure prediction method version IV. *Methods Enzymol* **266**, 540–553.
38 Levitt M & Chottia C (1976) Structural patterns in globular proteins. *Nature* **261**, 552–558.
39 Cuff JA, Clamp ME, Siddiqui AS, Finlay M & Barton GJ (1998) JPred: a consensus secondary structure prediction server. *Bioinformatics* **14**, 892–893.
40 Baker D & Sali A (2001) Protein structure prediction and structural genomics. *Science* **294**, 93–96.

41 Sträter N & Lipscomb WN (1995) Transition state analogue 1-leucinephosphonic acid bound to bovine lens leucine aminopeptidase: X-ray structure at 1.65 Å resolution in a new crystal form. *J Biol Chem* **34**, 9200–9210.
42 Taylor A, Peltier CZ, Torre FL & Hakaman N (1993) Inhibition of bovine lens leucine aminopeptidase by bestatin: number of binding sites and slow binding of the inhibitor. *Biochemistry* **32**, 784–798.
43 Kim H & Lipscomb WN (1993) X-ray crystallographic determination of the structure of bovine lens leucine aminopeptidase complexed with amastatin: formulation of a catalytic mechanism featuring a gem-diolate transition state. *Biochemistry* **32**, 8465–8478.
44 Vallee BL & Galdes A (1984) The metallobiochemistry of zinc enzymes. *Adv Enzymol Relat Areas Mol Biol* **56**, 283–430.
45 Vallee BL & Auld DS (1990) Active-site zinc ligands and activated H_2O of zinc enzymes. *Proc Natl Acad Sci USA* **87**, 220–224.
46 Sedmak JJ & Grossberg SE (1977) A rapid sensitive and versatile for protein using Coomassie Brilliant Blue G-250. *Anal Biochem* **79**, 544–552.
47 Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
48 Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci* **121**, 404–427.
49 Lund O, Nielsen M, Lundegaard C & Worning P (2002) CPHmodels 2.0: X3M, a computer program to extract 3D models. Abstract A102 presented at the CASP5 conference.