

The Structure and Enzyme Characteristics of a Recombinant Leucine Aminopeptidase rLap1 from *Aspergillus sojae* and Its Application in Debittering

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Abstract A leucine aminopeptidase Lap1 was cloned from *Aspergillus sojae* GIM3.30. The truncated Lap1 without a signal peptide was over-expressed in *P. pastoris*, and the enzymatic characteristics of recombinant Lap1 (rLap1) were tested. The rLap1 was about 36.7 kDa with an optimal pH 8.0 and optimal temperature 50 °C for substrate Leu-p-nitroanilide and it sustained 50 % activity after 1 h incubation at 50 °C. The activity of rLap1 was significantly inhibited by EDTA, whereas Co²⁺, Mn²⁺, and Ca²⁺ ions, but not Zn²⁺ ions, restored its activity. rLap1 showed the highest activity against Arg-pNA and then Leu-, Lys-, Met-, and Phe-pNA. The 3D structure of rLap1 showed it had a conserved functional charge/dipole complex and a hydrogen bond network of Zn2-D179-S228-Q177-D229-S158 around its active center. An acidic Asp residue was found at the bottom of the substrate binding pocket, which explains its preference for basic N-terminal amino acid substrates such as Arg and Lys. rLap1 improved the degree of hydrolysis of casein and soy protein hydrolysates and also decreased their bitterness, indicating its potential utility in food production.

Keywords *Aspergillus sojae* · Recombinant leucine aminopeptidase · *Pichia pastoris* · Debittering · 3D structure

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Introduction

Amino peptidases are a diverse set of peptidases that cleave residues from the amino (NH₂) terminus of peptides. Amino peptidases have a number of important biological functions, including roles in cell maintenance, growth, development, and defense [1, 2]. Depending on the number of amino acids cleaved from the NH₂ terminus of its substrates, amino peptidases can be classified as either amino peptidases, diaminopeptidases, or triaminopeptidases, all of which belong to E.C.3.4.11–3.4.13.a [3]. Their names are usually derived from the amino acids they preferentially cleave (for example, leucine amino peptidases, cystinyl amino peptidases, etc.), although other residues may also be cleaved [4–6]. With the exception of some cysteine and serine peptidases, the majority of the amino peptidases are metallo-enzymes and are assigned to six of 15 metalloproteases clans, designated as MA, MF, MG, MH, MN, and MQ [7]. Enzymes within these six metallo-amino peptidases clans usually contain one or two divalent metal ions (usually zinc, cobalt, manganese, etc.), bound to histidines in some conserved motifs, as ligands in their catalytic sites [8, 9].

In the food industry, leucine amino peptidases isolated from bacteria (such as *Bacillus* spp., *Aeromonas* spp., *Streptomyces* spp.) and fungi (such as *Aspergillus* spp.) are widely used in protein hydrolysate debittering, as they remove hydrophobic amino acids from the N-terminus of polypeptides, which contribute to their bitterness [10–12]. Amino peptidases are also widely used in cheese ripening and flavor development for a wide range of protein substrates [13, 14].

Amino peptidases from *Aspergillus oryzae* and *Aspergillus sojae* are regarded as food safe enzymes and have a long history of use in reducing bitterness. Four *A. oryzae* extracellular leucine amino peptidases (LAP), known as LAP I, II, III, and IV, were purified and enzymatically characterized in the 1970s [15, 16]. In 2002, Chien et al. purified Lap1 from *A. sojae* and identified its complementary DNA (cDNA) sequence to be 1.3 kb, encoding a 377 amino acid protein [17]. Until now, no report on the exogenous expression of the *A. sojae* Lap1 and its application in food processing has been published. Within the *A. oryzae* genome sequence, 19 amino peptidases have been identified [18]. *A. sojae*'s genome was also published in 2011, and it has 81.7 % similarity with that of *A. oryzae*, and these two *Aspergillus* species have similar proteases composition in their genomes [19]. In 2011, *lapA* from *A. oryzae* was cloned and over-expressed in *A. oryzae* RIB40 [20]. The *A. oryzae* LapA was identified as one of the two most important enzymes in the production of the antihypertensive peptide IPP from casein, as it processes its N-terminus in cooperation with another *A. oryzae* neutral protease, NpI, which processes its C-terminus [21]. LapA has 97 % identity to *A. sojae* Lap1; a difference of only 12 amino acids (six in the propeptide and six in the mature enzyme of LapA compared to Lap1). However, these two highly similar amino peptidases exhibit differences in substrate preference, although, whether the six different amino acids in mature protein are responsible for this altered substrate preference is still unknown.

To our knowledge, no report has so far analyzed the 3D structure of the leucine amino peptidases used in the food industry. This lack of information has inhibited our understanding of these enzymes' catalytic mechanisms and their substrate preferences. LapA and Lap1 belong to peptidase family M28 of clan MH. The amino peptidase AAP from *Aeromonas proteolytica* is a model enzyme within the MH clan, whose structure has been intensively studied for decades [22]. In this family, two co-catalytic metal ions (I

and II) are sequestered from bulk water in complex with conserved ligands in the order of His (II), Asp (I and II), Glu(I), Asp or Glu (II), and His (I) [23]. Two other residues, an Asp and a Glu in the motifs His-Xaa-Asp and Glu-Glu are also believed to be important for catalysis [24].

In this work, we have cloned the *lap1* cDNA from *A. sojae* GIM3.30, which encodes a protein which differs at three residues from the previously reported Lap1. We over-expressed *lap1* in *Pichia pastoris* which has been widely used in fungi aminopeptidases expression [25]. The enzyme characteristics of recombinant Lap1 (rLap1) were analyzed. Aminoacyl-p-nitroanilide (aminoacyl-pNA) substrates were used to test the substrate preference of rLap1. To analyze its catalytic mechanism and substrate preference, the 3D structure of rLap1 was reconstructed by Modeller using AAP as a model and its substrate-docking analysis was applied by Discovery Studio. Casein and soybean protein were chosen as animal and vegetable protein substrates to test rLap1's hydrolysis and debittering efficiency. Its potential use in food industry is also discussed.

Material and Methods

Chemicals

Chemicals used in this study were obtained commercially: Leu-pNA and Bestatin (Sigma–Aldrich China, Guangzhou, China), Ala-pNA, Lys-pNA, Phe-pNA, Met-pNA, Arg-pNA, Val-pNA, and Ile-pNA (GL Biochem, Shanghai, China). Other chemicals used in this study were of certified reagent grade.

Strains, Plasmids, Enzymes, Reagents

A. sojae GIM3.30 was maintained in our microbiology laboratory. *P. pastoris* KM71 strain (*his⁻*, *mut^s*), pPIC9K plasmid vector were from Invitrogen (California, USA). *E. coli* DH5 α , pMD18-T vector, and enzymes for manipulating DNA or RNA were purchased from Takara (Dalian, China). Primers used in this study were obtained from Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). All the protocols and culture media for *P. pastoris* KM71 strain were according to the *Pichia* Expression Kit manual (Invitrogen, California, USA).

cDNA Cloning of *lap1*

Total RNA of *A. sojae* was isolated from mycelia grown at 30 °C for 36 h in PDA solid culture medium (20.0 % potato extract, 2.0%D-glucose, 2.0%agar) using RNAiso Plus (Takara, Dalian, China) in accordance with the manufacturer's instructions. The cDNA pool was constructed with the PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio Inc.). The cDNA of *lap1* was amplified with the PrimeSTAR HS DNA Polymerase and primer1, 5'-ATGCGT TTCCTCCCCTGC-3', primer2, 5'-TTACAGTGAATCTGCGAA-3', were designed on the basis of the sequence reported in GenBank (accession no. AF419160.1). The purified PCR product was cloned into pMD18-T and then transformed into *Escherichia coli* DH5 α competent cells. The transformants (pMD18-T/*lap1*) were screened on an LB plate (Amp^r). The pMD-18 T/*lap1* vector was fully sequenced.

Construction of Recombinant *lap1* Expression Vector and Transformation of Recombinant *P. pastoris*

The identified pMD18-T/*lap1* vector was used as a template. The truncated *lap1* gene (without signal peptide encoding sequence) was amplified by PCR using primer3 (5'-CGCGAATTCATTGGAGACCATGTACGC-3') and primer4 (5'-AATGCGGCCGCTTACAGCGAATCTGCGAA-3'). The underline indicates the *EcoRI* and *NotI* restriction sites, respectively. The PCR product was purified and cut with *EcoRI* and *NotI* and then ligated with the pPIC9K vector. The pPIC9K/*lap1* vector was transformed to *E. coli* DH5 α , and transformants were screened on an LB plate (Amp^r). Finally, the pPIC9K/*lap1* vector was identified by PCR, restriction digestion, and sequencing. The pPIC9K/*lap1* vector was linearized with *SaII*, and transformed into *P. pastoris* KM71 by electroporation (Electroporator 2510, Eppendorf, Hamburg, Germany), with the following conditions: 2 mm electroporation cuvette, 1500 V, 4–6 ms. The strain transformed with empty pPIC9K vector was used as a control. Following pulsing, 0.8-ml ice-cold 1.0 M sorbitol was added to the electroporation cuvette and incubated in ice water for 5 min. The transformants were then spread on MD minimal medium plates and grown at 30 °C until single colonies appeared (3–4 days). To verify *lap1* gene integration into the genome of *P. pastoris* KM71, transformants were identified by genomic PCR test using two pairs of primers, primer3/4 and α -Factor/3'AOX1 primers (α -Factor, 5'-TACTATTGCCAGCATTGCTGC-3' and 3'AOX1, 5'-GGCAAA TGGCATTCTGACATCC-3').

Expression of *lap1* in Triangular Flask

The recombinant *P. pastoris* KM71 was inoculated to 25 ml BMGY media in a 250 ml Erlenmeyer flask and shaken (200 rpm) for approximately 24 h at 30 °C until log phase growth was reached (OD600 = 5–6). The yeast cells were harvested by centrifugation at 5000 g for 10 min at room temperature and then resuspended in 50 ml BMMY media in 500 ml Erlenmeyer flask. The initial OD of the BMMY culture was between 2.0 and 4.5. The flask was shaken (250 rpm) at 28 °C for 6 days. The expression of rLap1 was induced by the addition of 500 μ l methanol every 24 h. The supernatant of the fermentation broth was collected at different time points (every 24 h) by centrifugation at 10,000 g for 5 min at 4 °C. The wet weight of yeast cells was recorded. SDS-PAGE was performed and the gel was stained by 0.2 % Coomassie Brilliant Blue (CBB) R-250.

Purification of rLap1

The 6-day fermentation broth supernatant was collected by centrifuging the culture at 10,000 g for 10 min at 4 °C. After dialysis in 20 mM Tris-HCl buffer (pH 8.0) at 4 °C overnight, the supernatant was filtered through a 0.22 μ m filter and collected as the crude aminopeptidase solution. This solution was concentrated using an Amicon Ultra-15 10 K molecular weight cutoff centrifugal filter (Millipore, Billerica, MA, USA) in 20 mM PBS buffer (pH 7.5) and then applied to a Sephadex G-75 column (GE Healthcare, Buckinghamshire, UK) with 20 mM PBS buffer (pH 7.5). The active fractions were pooled for additional analysis.

Aminopeptidase Activity Assay

Leucine aminopeptidase activity was determined with an artificial substrate, Leu-pNA as the protocol describe before with some modifications [26]. A standard reaction was performed in 50 mM Tris–HCl (pH 8.0) containing 1 mM Leu-pNA and an appropriate amount of the enzyme. The reaction mixture was incubated at 50 °C (the optimal temperature of rLap1) for 20 min and terminated by adding an equal volume of 20 % (v/v) acetic acid at predetermined times. The absorbance at 405 nm was measured with a Mini 1240UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan) to determine the amount of pNA liberated. The enzyme activity unit (U) was defined as the amount of enzyme that produced 1 µg of pNA per min. The activity of rLap1 against different substrates (Ala-pNA, Lys-pNA, Phe-pNA, Met-pNA, Arg-pNA, Val-pNA, and Ile-pNA) was assayed using the same conditions.

Biochemical Analysis

Purified rLap1 was used to determine the optimum temperature, pH, thermal stability, and metal ion dependency of the enzyme.

Effect of Temperature and pH Aminopeptidase activity assays were performed at various temperatures ranging from 0 to 90 °C to determine the temperature optimum for rLap1. Similarly, the enzyme assays were carried out at different pH values from 3.0 to 11.0.

Temperature Stability Thermal stability of the enzymes was determined according to the protocol of Karadzic et al. [27]. The enzymes were incubated in Tris–HCl buffer (pH 8.0) at various temperatures (25, 50, 60, 65, 70, 75, 80, and 90 °C) for various time intervals (0, 20, 40, 80, and 120 min). After heat treatment, samples were cooled and the enzyme was subjected to aminopeptidase activity assay at 50 °C for 20 min.

Effects of Inhibitors and Metal Ions on rLap1 Activity Two protease inhibitors (EDTA and Bestatin) were added to the reaction mixture to study their effects on enzyme activity. The enzyme was incubated at 30 °C in Tris–HCl buffer (pH 8.0) with 1 mM Bestatin and 10 mM EDTA for 20 min before enzyme assay. Seven different metal ions (CaCl₂, CoCl₂, CuCl₂, NiCl₂, MgCl₂, MnCl₂, ZnCl₂) were used to analyze their ability to restore rLap1 activity after inhibition by a chelating agent. After the removal of EDTA by Centricon YM-10 filter membrane (Millipore), a 1 mM divalent metal salt solution was added, and incubation was continued for an additional 20 min before the enzyme assay. Enzyme assays was carried out at 50 °C for 20 min as described above. The enzyme activity of recombinant rLap1 without the addition of inhibitors was defined as 100 %. The effects of metal ions and protease inhibitors on rLap1 activity were calculated as the mean and standard error of three independent experiments.

Hydrolysis and Debittering

Casein and soy protein isolates (SPI) were dissolved in 20 mM phosphate buffer (pH 7.5 and 8.5) and hydrolyzed by recombinant *A. oryzae* neutral protease I (rNpI, 8000 U/g substrate) produced in our lab or Alcalase (8000 U/g substrate), respectively [28]. The reactions were

performed at 50 °C for 6 h. After digestion, hydrolysates were heated at 100 °C for 10 min to inactivate the enzymes. Then, Casein and SPI hydrolysates were further hydrolyzed by rLap1 (8000 U/g) in 20 mM phosphate buffer (pH 8.5) at 50 °C for 6 h.

The degree of hydrolysis (DH) is defined as the percentage of free amino groups cleaved from protein, which is calculated from the ratio of α -amino nitrogen and total nitrogen. The α -amino nitrogen was determined by a modified formal titration method. A hydrolysate sample in a volume of 5 mL was added to 60 mL distilled water, which was then adjusted to pH 8.2 using 0.1 M NaOH. After 20 mL of previously neutralized 38 % (v/v) formaldehyde solution was added, the mixture was titrated with 0.1 M NaOH to pH 9.2, which was standardized against potassium hydrogen phthalate. Total nitrogen was determined by the BCA method.

The bitterness score of the hydrolysate was determined quantitatively by a bitterness expert panel of 9 people and expressed as caffeine equivalent (CE) value [29]. The samples were scored on a scale of 0–10 calibrated with 0–1000 ppm of CE. A set of three samples was presented to the panel as well as the caffeine standard solutions at 0, 125, 250, 500, and 1000 ppm. The panel tasted the samples in comparison with the caffeine solutions and scored them on the scale accordingly.

Homology Modeling and Molecular Docking

The three dimensional structure of mature rLap1 was modeled by homology modeling against the structure of AAP (PDB: 1LTQ) using the program MODELLER version 9.12 [30, 31]. The identity of rLap1 to AAP was 31.7 %. The protein model was optimized using energy minimization with the discovery module of Discovery Studio Package 3.5 (Discovery Studio 3.5, Accelrys, Co. Ltd.). The program was also used to view the structure and to calculate the atomic distances at the active site of rLap1.

The steroid substrates (L-Xaa 4-nitroanilide) were retrieved in MDL/SDF format, and charges were assigned according to the CHARMM molecular force field [32]. Molecular docking of substrates in the active site of the rLap1 model was performed by Discovery Studio 3.5. The residues His⁹⁹, Asp¹¹⁸, Glu¹⁵⁷, Asp¹⁸⁴, and His²⁶⁶ were selected as part of the metal binding site. The backbone was kept rigid during the docking calculations.

Results and Discussion

Cloning of rLap1 from *A. sojae* and Expression Vector Construction

The cDNA of *lap1* from *A. sojae* GIM3.30 was cloned into pMD18-T vector. The *lap1* sequence from *A. sojae* GIM3.30 was 99.7 % identical to the *lap1* previously reported (GenBank accession no: AF419160.1) [17]. Three base substitutions (T458C, A574G, and T743C) were present in the sequence we cloned, leading to mutations in the mature peptide at F76S, S115G, and L171S. It is interesting to note that these three mutations in the Lap1 we cloned were exactly the same as their counterparts in the LapA sequence from *A. oryzae*. The Lap1 we cloned from *A. sojae* GIM3.30 seems to be an intermediate between LapA and Lap1. LapA was reported to have six amino acid differences from Lap1 in its mature region, supposedly leading to their different substrate preferences. In the mature rLap1, the conserved metal coordinated ligands of His⁹⁹ (II), Asp¹¹⁸ (I and II), Glu¹⁵¹ (I), Asp¹⁸⁴ (II), and His²⁶⁶ (I)

were found. The two conserved His-Xaa-Asp and Glu-Glu motifs, important for catalysis in M28 family peptidases, were found as His⁹⁹-Gln¹⁰⁰-Asp¹⁰¹ and Glu¹⁵¹-Glu¹⁵² in the mature rLap1 (Fig. 1). Compared with other bacteria and fungi aminopeptidases in M28 family, these metal ligands and catalytic His-Xaa-Asp and Glu-Glu motifs are highly conserved (Fig. 1).

A truncated *lap1* gene without a signal peptide, obtained from pMD18-*T/lap1*, was cloned into pPIC9K downstream of the vector’s α-factor signal site. After verification by sequencing, the expression vector pPIC9K/*lap1* was transformed into *P. pastoris* KM71. PCR screening of the transformants showed that *lap1* had been integrated into the genome of *P. pastoris* KM71.

Expression and Purification of the rLap1 in *P. pastoris*

Recombinant Lap1 production in *P. pastoris* was induced by the addition of methanol. SDS-PAGE analysis of the fermentation supernatant collected at day 6 from 500 mL Erlenmeyer flasks is shown in Fig. 2. In this gel, an rLap1 band was seen in the recombinant *P. pastoris* supernatant, in contrast with the empty vector control. After chromatographic separation using Sephadex G-75, a clear purified rLap1 band was seen on the gel (Fig. 2, lane 2). According to the plot of relative mobility versus molecular mass, purified rLap1 was calculated to be 36.7 kDa. The putative molecular weight of the recombinant truncated rLap1 without its own signal peptide is about 40.3 kDa. The purified rLap1 is most likely to be the mature enzyme without its propeptide. Its molecular weight was similar to that of the mature wild-type Lap1 isolated from *A. sojae* whose molecular weight is about 37 kDa [17]. The enzyme activity reached 776.9 U/mL in the fermentation broth supernatant after 120 h of culture in 500 mL Erlenmeyer flasks. The enzyme yield is comparatively high in the Erlenmeyer flask fermentation, and we believe that with further optimization of the culture conditions and utilization of batch and fed-batch fermentation, rLap1 production would be high enough for its use in the food industry.



Fig. 1 Alignment of the rLap1 mature peptidase with *Vibrio proteolyticus* aminopeptidase (AAP, IRTQ_A), *Aspergillus niger* aminopeptidase 1 (AnLap1, XP_001400691), and *Aspergillus oryzae* aminopeptidase A (AoLapA, XP_001825745) sequences. Identical amino acids are shown as gray background. The motifs for the catalysis (His99-Xaa100-Asp101 and Glu151-Glu152) are indicated by red open rectangle. Green arrows indicate putative Zn ligands. Residues involved in forming the hydrophobic binding pocket are indicated by yellow diamonds. Residues involved in forming the hydrogen network Zn2-D184-S238-Q182-D239-S163 indicated by blue stars

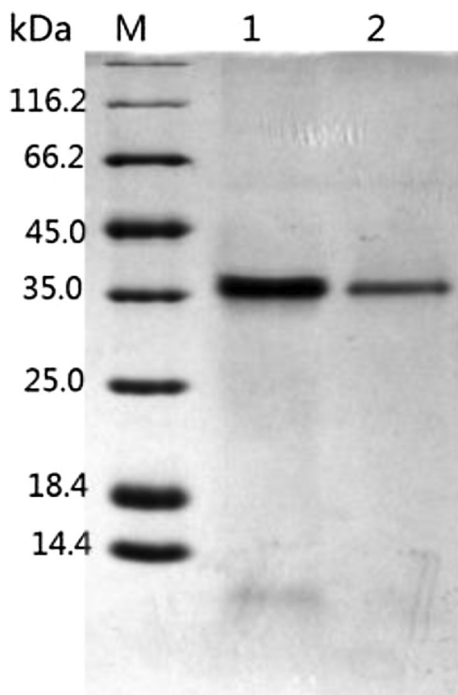


Fig. 2 SDS-PAGE analysis of fermentation supernatant and purified rLap1. Lanes: M, protein molecular weight markers; 1, supernatant of *P. pastoris* (pIC9K/lap1); 2, rLap1 purified by Sephadex G-75 chromatography

Biochemical Characterization of the rLap1

The pH and temperature effects on the activity of rLap1 are shown in Fig. 3. Its optimum pH was approximately 8.0 (Fig. 3a), which is similar to isolated *A. sojae* Lap1 and over-expressed *A. oryzae* LapA. Between pH 7–9, rLap1 enzyme activity remained above 70 %. At pH 6 and 10, its activity was quickly lost. Both Lap1 and LapA lost their activity at pH 6, but Lap1 maintained 80 % activity at pH 10 and LapA showed more than 80 % activity at pH 11. rLap1 had an optimal temperature of 70 °C (Fig. 3b) similar to the previously reported Lap1, while LapA's temperature optimum was 60 °C. After a 2-h incubation at 60 °C, rLap1 maintained above 90 % activity and above 50 % activity after a 2-h incubation at 70 °C, which shows it possesses good thermal stability (Fig. 3c). Lap1 was only able to sustain 50 % activity after 1 h of incubation at 50 °C and LapA was inactivated at 70 °C. We are not sure if the amino acid differences between these enzymes are responsible for the differences in their pH and temperature optima.

The protease inhibitor assay showed that rLap1 was a typical metallo-aminopeptidase, as it was significantly inhibited by bestatin and the chelating agent EDTA (Table. 1). After the removal of the chelating agent, a divalent metal salt solution was added. The activity of rLap1 was recovered the best by 1 mM Co^{2+} and then Mn^{2+} and Ca^{2+} . It is surprising to find that the enzyme activity was unable to be restored by Zn^{2+} , which is believed to be the most common metal in the active site of aminopeptidases. We have previously seen that the addition of Co^{2+} to the culture medium can improve the activity of rLap1; however, this did not occur with other divalent metal ions (unpublished data). In a similar assay, the activity of Lap1 from

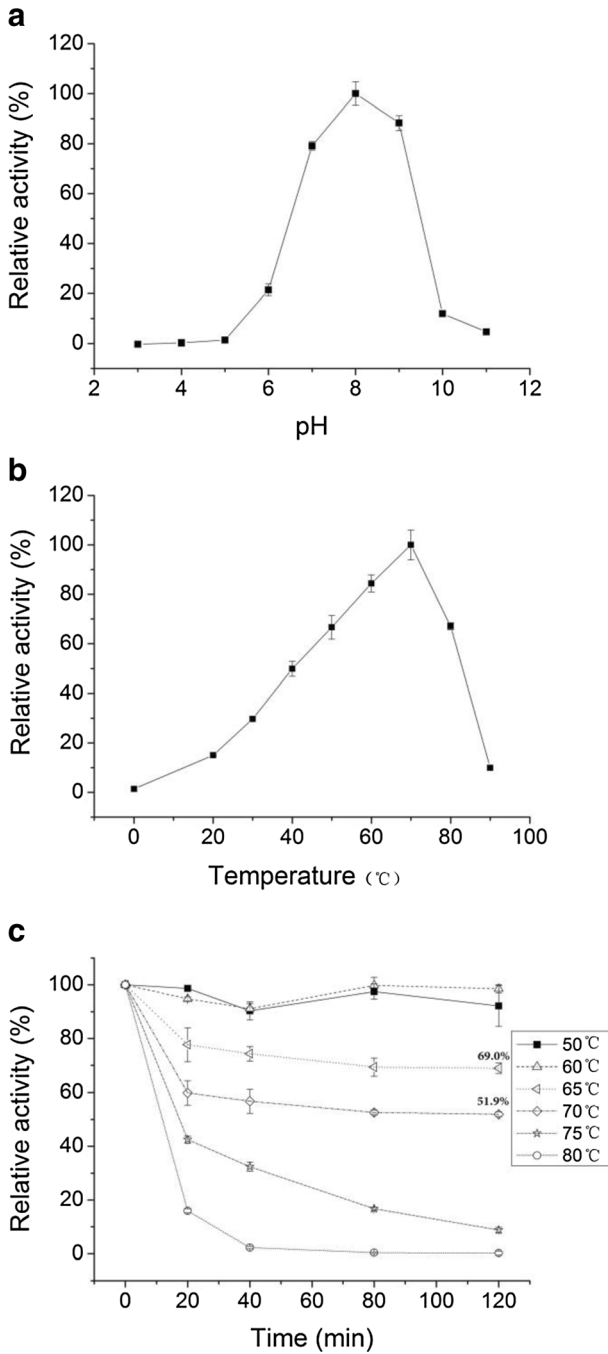


Fig. 3 Effect of pH and temperature on activity and stability of rLap1: **a** effect of pH on the activity of rLap1; **b** effect of temperature on the activity of rLap1; **c** effect of temperature on the stability of rLap1. For the effect of temperature, the buffer used was 50 mM Tri-HCl (pH 8.0)

Table 1 Effects of various chemical compounds on rLap1 hydrolysis of Leu-pNA

Compound (mM)	Relative activity (%)
None	100
^a Bestatin (1 mM)	2.88
^a EDTA (10 mM)	4.35
^b EDTA (10 mM) + CoCl ₂ (1 mM)	37.64
^b EDTA (10 mM) + MnCl ₂ (1 mM)	16.57
^b EDTA (10 mM) + CaCl ₂ (1 mM)	12.69
^b EDTA (10 mM) + MgCl ₂ (1 mM)	6.61
^b EDTA (10 mM) + NiCl ₂ (1 mM)	2.96
^b EDTA (10 mM) + ZnCl ₂ (1 mM)	1.02
^b EDTA (10 mM) + CuCl ₂ (1 mM)	0.47

^a The enzyme was incubated at 30 °C in Tris–HCl buffer (pH 8.0) with 1 mM Bestatin and 10 mM EDTA for 20 min before enzyme assay

^b After the removal of the chelating agent by Centricon YM-10 (Millipore), the divalent metal salt solution was added, and incubation was continued for an additional 20 min before the enzyme assay. Aminopeptidase activity assayed in the absence of inhibitors was taken as 100 %

A. sojae could be recovered best by Zn²⁺, and then Co²⁺ and Mg²⁺, following removal of the chelating agent. Zn²⁺ and Co²⁺ (at 1 mM each) were also reported to increase LapA activity to 258 and 122 %, respectively, relative to the control reaction. The reason why rLap1 does not recruit Zn²⁺ as its divalent metal is not currently known.

Substrate Preference of rLAP1

Substrate preference was tested using a variety of aminoacyl-pNA derivatives (Table 2). It appears that Arg-pNA (109.8 %) was most the efficiently hydrolysed substrate, even higher

Table 2 Substrate specificity of the purified rLap1, Lap1, and LapA

Substrate ^a	Relative	Activity(%) ^b	
		rLap1	Lap1 [18]
Leu-pNA	100.0	100.0	100.0
Arg-pNA	109.8	20.3	16.5
Lys-pNA	76.7	41.3	24.5
Met-pNA	74.5	–	29.6
Phe-pNA	63.8	98.6	38.8
Ile-pNA	15.3	–	3.1
Tyr-pNA	10.0	–	–
Ala-pNA	3.9	4.4	1.2
Pro-pNA	1.6	0.5	1.2–3.1

– not detected

^a All substrates were used at a final concentration of 1 mM

^b The rate of hydrolysis is expressed as a percentage relative to the activity obtained by using Leu-pNA as the substrate (set at 100 %)

than Leu-pNA (relative activity set to 100 %). Another positively charged amino acid, Lys-pNA (76.7 %), and two hydrophobic aminoacyl-pNAs, Met-pNA (74.5 %) and Phe-pNA (63.8 %), were also good substrates, while hydrolytic activity against Ile-pNA (15.3 %) and Tyr-pNA (10.0 %) was low. Ala- and Pro-pNA were practically resistant to the action of the enzyme. The substrate preferences of Lap1 and LapA are also shown in Table 1. Similarly, Lap1 and LapA showed that they preferred bulky hydrophobic and positively charged amino acids as substrates, but the relative activity of each amino acid differed greatly among rLap1, Lap1, and LapA. Chien et al. suggested that the six amino acid differences in the mature region between Lap1 and LapA might be responsible for their difference in substrate preference [17]. There are three amino acid differences each between Lap1 and rLap1 (Lap1 Phe⁷⁶Ser, Ser¹¹⁵Gly, and Leu¹⁷¹Ser) and LapA and rLap1 (LapA Lys¹⁸Gln, Asp¹⁰⁶Gln, and Val²¹⁸Ile). These six amino acids are far from the active site and substrate pocket (see the reconstructed rLap1 3D structure below). Whether or how these amino acid substitutions affect substrate preference is not known.

3D Homology Modeling of rLap1

To understand rLap1's catalytic mechanism and substrate preference, 3D homology modeling was performed against the model M28 family aminopeptidase AAP from *Vibrio proteolyticus* (the enzyme to which rLap1 has the highest identity). Although the mature rLap1 and AAP have only 31.7 % identity, the 3D reconstruction of rLap1 was well superimposed on AAP, which is in accordance with the principle of proteins having greater conservation of their tertiary structures than their primary sequence (Fig. 4a).

In its active center, the two divalent metal ions coordinated with His⁹⁹ (II), Asp¹¹⁸ (I and II), Glu¹⁵¹ (I), Asp¹⁸⁴ (II), and His²⁶⁶ (I) forming a functional charge/dipole complex (Fig. 1). Beside these five conserved first-shell residues, another conserved Glu¹⁵² in the Glu-Glu motif was also found in rLap1 (Fig. 4b). This Glu is thought to function as a proton acceptor from the amino-terminus of the incoming peptide and, subsequently, the proton donor to the newly formed amino-terminus, which is a rate-limiting step during catalysis [22]. Mutation of Glu¹⁵² rendered AAP inactive [33]. Apart from the first-shell residues, the conserved second-shell residues outside of the charge/dipole complex are thought to support the electronic strain of the complex produced as a consequence of functional polarization. A hydrogen bond network of Zn2-D179-S228-Q177-D229-S158 outside the first-shell residues was found to be conserved in AAP and another model enzyme SGAP (*Streptomyces griseus* aminopeptidase), although they share only 25 % sequence identity [34]. Mutation of the AAP second-shell residue S228A caused a tenfold loss of activity. In this study, this hydrogen network Zn2-D184-S238-Q182-D239-S163 was also found in rLap1 (Fig. 1).

All eight residues in the substrate binding pocket were conserved in rLap1, Lap1, and LapA. Six of the eight residues in rLap1, including Met¹⁸⁵, Tyr²³⁵, Cys²³⁷, Phe²⁵⁴, Phe²⁵⁸, and Ile²⁶⁵, were also conserved in AAP (Figs. 1 and 4c). Met²⁴² in AAP was substituted by another hydrophobic residue Phe²⁵². These hydrophobic residues in the binding pocket explain the ability of AAP, rLap1, Lap1, and LapA to efficiently remove bulky hydrophobic residues. Figure 5c shows the docking of Phe in the rLap1 substrate pocket, while a smaller Ala residue does not fit in this large pocket (Fig. 5a). The most significant difference between the substrate binding pockets of AAP and rLap1 was the residue Tyr²⁵¹ in AAP substituted by an acidic Asp²⁶¹ in rLap1 (Fig. 5b). This negatively charged residue was right at the bottom of the rLap1 substrate pocket. This finding explains the preference for positively charged substrates (such as

Fig. 4 The rLap1 model superimposed on AAP (1LTQ.pdb). **a** The full view of rLap1 model (yellow) and AAP structure (cyan). **a** red circle highlights the activation center. **b** The metal binding site of rLap1 (carbon atoms in black) and AAP (carbon atoms in white). **c** The residues of rLap1 (yellow) and AAP (cyan) involved in forming the hydrophobic substrate binding pocket

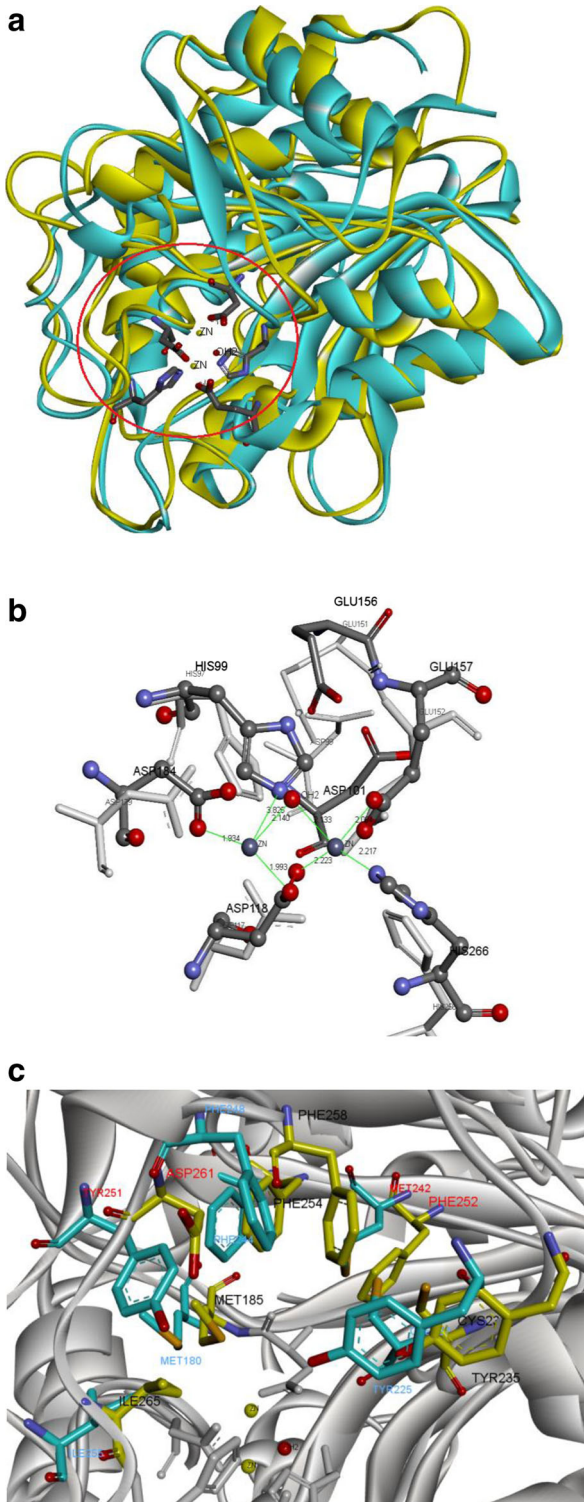
Arg and Lys) of rLap1, Lap1, and LapA. Figure 5b shows the docking of Lys in rLap1. The electrostatic interaction and hydrogen bond between the negative side chain of Asp²⁶¹ and that of the incoming positive Lys accommodates Lys well within the pocket. Without this negative residue in the bottom of the binding pocket, AAP showed no activity against N-terminal Arg and Lys residues [35].

Hydrolysis and Debittering

To test the efficiency of rLap1 in the proteins hydrolysis and hydrolysates debittering, casein and soybean protein isolate (SPI) hydrolysates catalyzed by Alcalase or rNpI were used as animal and vegetable protein substrates. The DH of casein hydrolysates catalyzed by Alcalase increased from 10.5 to 23.9 % (Fig. 6a), and its bitterness was reduced from an average score of 2.9 to 1.1 after further digestion by rLap1 for 6 h (Fig. 6b). The DH of casein hydrolysates catalyzed by rNpI was improved from 7.7 to 18.1 %, and its bitterness was reduced from a score of 6.1 to 2.4. The DH of SPI hydrolysates catalyzed by Alcalase was improved from 19.1 to 23.6 %, and its bitterness score was reduced from 4.8 to 1.6. All these results showed that rLap1 was efficient at protein hydrolysis and debittering. Due to its substrate preference, rLap1 could efficiently remove N-terminal Leu, Met, and Phe, which contributes to its debittering effect. However, we also noted that rLap1 had low activity against N-terminal Pro, another important contributor to bitterness in polypeptides, which may lead to a low debittering effect on proline-rich peptides. Even so, the high efficiency of rLap1 in both hydrolysis and debittering of vegetable and animal protein hydrolysates suggests that it has great potential to be used in the food industry for protein processing.

Another potential use of rLap1 is in the production of antihypertensive peptides. *A. oryzae* LapA and neutral protease I (NpI) have been identified as the major enzymes responsible for the production of the antihypertensive peptide IPP from casein. We have efficiently expressed *A. oryzae* rNpI in *P. pastoris*, and rLap1 has similar substrate preference to LapA. In this study, we have shown that rNpI and rLap1 can efficiently hydrolyze casein and other recent results have shown that this hydrolysate could efficiently inhibit the activity of angiotensin I converting enzyme (ACE) (unpublished data). We are now in the process of isolating and identifying the antihypertensive peptides in this hydrolysate.

In conclusion, we have produced a recombinant form of the *A. sojae* aminopeptidase Lap1 in *P. pastoris*. The 3D reconstruction of rLap1 has shown its conserved structure-function relationship with aminopeptidase family M28, and the structure of its substrate pocket explains well its preference for hydrophobic and basic N-terminal amino acids. Vegetable and animal protein hydrolysates were efficiently hydrolyzed and debittered by rLap, suggesting it has great potential to be used in protein processing within the food industry. rLap1 and rNpI also have the potential to produce antihypertensive peptides. Our further work will exploit the hydrolysis and debittering abilities of rLap1 in different food protein sources. The antihypertensive peptides produced by rLap1 will be further isolated.



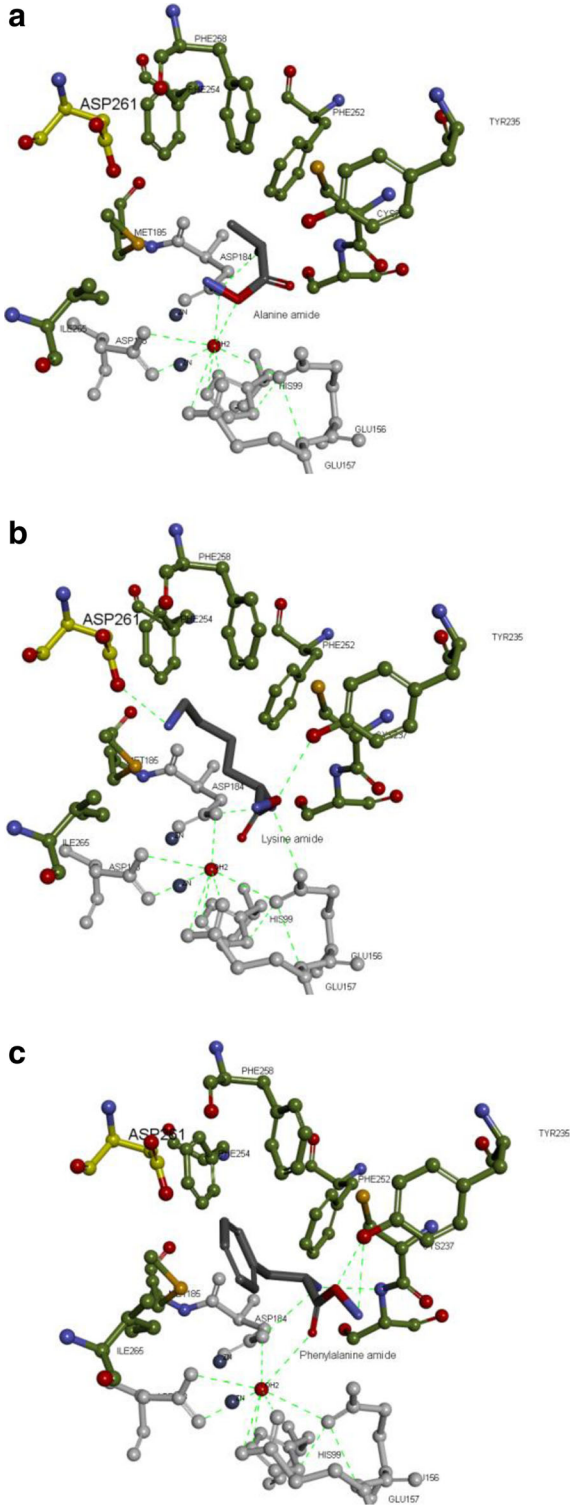


Fig. 5 rLap1 hydrophobic substrate binding pocket (carbon of hydrophobic residues in green and the acidic Asp²⁶¹ in yellow) docking with Alanine-pNA(A), Lysine-pNA(B), and Phenylalanine-pNA(C) molecules (black carbon atoms). The substrate molecules just show the amino acid amide section

Abbreviations and Nomenclature

PCR, polymerase chain reaction; rNpI, recombinant neutral protease I; Lap1, Leucine aminopeptidase 1 from *A. sojae*; LapA, Leucine aminopeptidase A from *A. oryzae*; rLap1, recombinant Leucine aminopeptidase 1; AAP, aminopeptidase from *Aeromonas proteolytica* (*Vibrio proteolyticus*); AOX, gene encoding alcohol oxidase; MD, minimal medium containing dextrose; OD, optical density; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; YPD, yeast extract-peptone-dextrose medium; pNA, p-nitroanilide; SDS-PAGE, sodium dodecyl sulfate

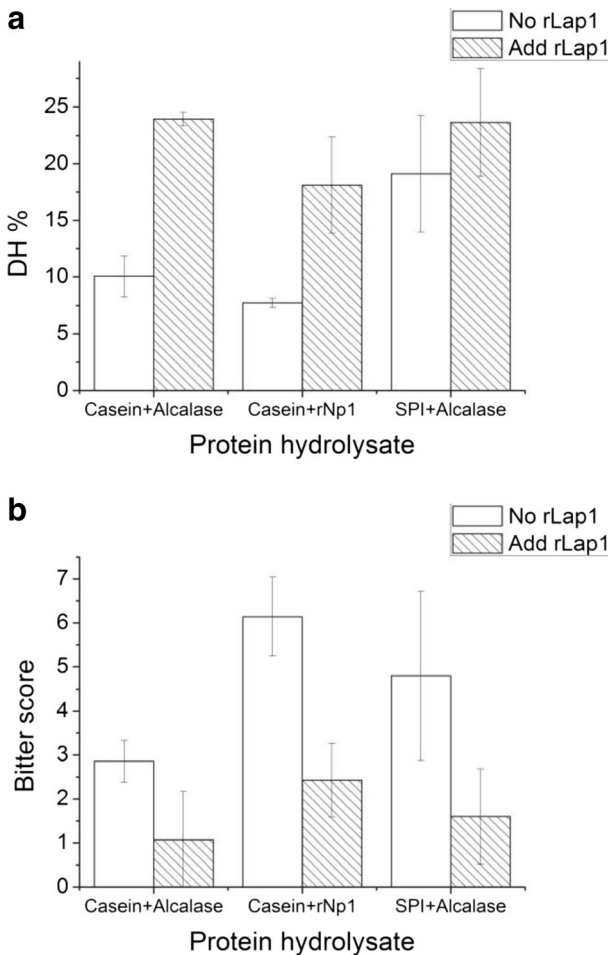


Fig. 6 The hydrolysis and debittering effects of rLap1 on casein and SPI protein hydrolysates. **a** The DH of casein and SPI hydrolysates catalyzed by rLap1. **b** The bitter score of casein and SPI hydrolysates treated with rLap1

polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DH, degree of hydrolysis; SPI, soybean protein isolate.

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