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Biochemical Properties of Recombinant Leucine Aminopeptidase II from *Bacillus stearothermophilus* and Potential Applications in the Hydrolysis of Chinese Anchovy (*Engraulis japonicus*) Proteins

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ABSTRACT: The effects of various factors on the activity and conformation of recombinant leucine aminopeptidase II (rLAP II) from *Bacillus stearothermophilus* and its potential utilization in the hydrolysis of anchovy proteins were investigated. The optimal temperature and pH of rLAP II were 55 °C and 8.0 in phosphate buffer, and its activity was strongly stimulated by Co^{2+}. Conformational studies indicated that maintaining the α-helical structure had a critical effect on rLAP II activity. rLAP II was used to hydrolyze anchovy proteins, and it exhibited high specificity for peptides with molecular weight between 6000 and 1000 Da and positive coordination with endogenous enzymes and commercial Flavourzyme. Its use will enhance protein hydrolysis in species of aquatic animals. rLAP II could potentially be used to remove bitterness in the protein hydrolysis industry.

KEYWORDS: *anchovy, Bacillus stearothermophilus, circular dichroism spectroscopy, hydrolysis, leucine aminopeptidase*

INTRODUCTION

For many years, high consumption of natural resources, production of large quantities of industrial waste and environmental pollution were characteristics of the food processing industry. Substantial quantities of animal byproduct and food waste materials are annually generated by manufacturing processes worldwide.[1,2] Nowadays with increasing awareness of the environment and the scarcity and ever increasing cost of land-fill space, better and more value-added utilization of the under-utilized byproducts of various industries has become an attractive means of reducing environmental problems and maximizing economic benefits.[3] In fact, by-products from the food and animal industries are rich in protein, fat, and vitamins, etc. These are very important bioresources that may be reutilized in feedstuffs, health-care products, and pharmaceuticals or as specialty feeds for fish and other animals, and even for human consumption.[4] *Engraulis japonicus* (anchovy) is one of the main swimming aquatic species in China. They are found in extensive areas throughout the Yellow Sea and Bohai large reserves, with an estimated annual harvest of 700−800 t.[5] They are usually taken as under-utilized byproducts in the fishery industry because of the ease of decay and their small bodies. Actually, anchovies are rich in protein (up to 15−20%) and possess abundant endogenous enzymes,[6] so they can be used as a raw material for the production of protein hydrolysate with high added value. Accordingly, effective utilization of these byproducts provides a path to sustainable development. Therefore, devising ways to better use these bioresources has become an important goal of all biological researchers.

Processes involving enzymatic hydrolysis of proteins are rapidly gaining ground due to the various advantages they offer over conventional chemical processes, especially with regard to the environment and costs.[7] Moreover, the functional properties and nutritional value of proteins can be improved by enzymatic hydrolysis,[8] which can generate polypeptides and free amino acids.[9,10] Polypeptides of low molecular weight can be absorbed by the intestine directly and are associated with low allergenic effects.[11] In addition to nutrient utilization, other biological functions of polypeptides have been reported, including antihypertension, opioid agonist or antagonist, immunomodulatory, antioxidant, anticancer, antithrombotic and antimicrobial activities.[12,13] For these reasons, a number of commercially available proteases, such as trypsin, Alcalase, and Neutrase,[14,15] have been employed to specifically modify proteins and produce peptides. The demand for enzymes with specific properties is high, and various enzyme sources are being investigated.[16] However, economic considerations often limit the type and quantity of enzymes that can be used in industrial protein hydrolysis. Therefore, the search for enzymes that can be broadly used for hydrolysis on an industrial scale has received more attention recently.

Aminopeptidases are a class of exopeptidase that selectively catalyzes the sequential removal of amino acids from the N-terminus of proteins and peptides. Leucyl aminopeptidases (LAPs; EC 3.4.11.1), which belong to Clan M and M17 families, were the first to be discovered and are the most widely studied aminopeptidases. LAPs prefer to cleave leucine residues from the N-terminus of proteins and peptides.[17] *Bacillus*

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stearothermophilus LAP II (LAP II) is a dimeric enzyme with an apparent molecular mass of 46 kDa that was first isolated by Motoshima et al.[18] Recently, the gene encoding LAP II was cloned and expressed at high levels in *Escherichia coli*, and the biochemical properties of the recombinant enzyme (rLAP II) were determined.[19] Despite this, the ways in which various factors (e.g., pH, temperature, and divalent cations) affect the enzymatic activity of rLAP II are still unknown. Furthermore, the biological significance of rLAP II, as well as the applied functions, is still not fully understood. In this article, the dependence of the enzymatic activity of rLAP II upon various factors (pH, divalent cations and temperature) is described. The effects of these factors on the secondary structure of rLAP II were also studied in order to try to understand the mechanisms that induce variations in enzymatic activity under different conditions. In addition, potential applications of rLAP II in the hydrolysis of fishery (anchovy) byproducts were also determined. The data presented here not only considerably expand general knowledge of this enzyme but also provide a basis for its further use in the food processing industry.

MATERIALS AND METHODS

Chemicals, Enzymes, and Strains. *E. coli* DH5α, *E. coli* BL21 (DE3), kanamycin, T4 DNA ligase, restriction endonucleases (*BamH I* and *Xho I*), and IPTG were purchased from Takara Co., Ltd. (Dalian, People's Republic of China). *Pfu* DNA polymerase was purchased from Promega (USA), and E.Z.N.A. Plasmid Mini kit I was purchased from Omega (USA). pBluescript SK and pET28a vector were purchased from Stratagene (La Jolla, CA). Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin was obtained from Qiagen Inc. (Valencia, CA, USA). Reagents for polyacrylamide electrophoresis such as acrylamide, bis-acrylamide, ammonium persulfate, TEMED, and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad (USA). All other chemicals used in the present study were of analytical grade and were purchased from Sigma Chemical Co. (USA).

Total Gene Synthesis of Leucine Aminopeptidase II and Construction of the Expression Plasmid. According to the sequence of the gene submitted to the NCBI (GenBank: D13385.1), 25 pairs of oligonucleotides (45−55-mer) were designed and synthesized. There was a 15-mer overlap for each of the oligonucleotides used, with the first and last oligonucleotides harboring a restriction endonucleases site of *BamH I* and *Xho I*, respectively. The full length of the gene template was acquired according to the PCR-based two-step DNA synthesis method of Xiong et al.[20] The object gene was cloned into the cloning vector pBluescript SK and was used to transform *E. coli* DH5α. The recombinant plasmids were extracted using a plasmid mini kit and sequenced to ensure sequence accuracy. Plasmids with the correct sequence were digested with the restriction endonucleases (*BamH I* and *Xho I*) and ligated into the expression vector pET28a that contained 6× His-tag to form pET28a $(His)_6$-LAP-$(His)_6$. The recombinant plasmid was used to transform *E. coli* BL21 (DE3).

Gene Expression and Enzyme Purification. To purify rLAP II, *E. coli* BL21 (DE3) cells that harbor pET28a-LAP were grown at 37 °C in 1 L of LB medium that contained 2 mL of 1.0 M kanamycin and induced at an optical density of 0.8 at 600 nm by IPTG to a final concentration of 0.05 mM. After 12 h of induction at 20 °C, the cells were harvested, resuspended in 100 mL of 20 mM Tris-HCl (pH 8.0), and disrupted by sonication (ULTRASONIC PROCESSOR UH-950A, AutoScience). The cell lysate was then centrifuged at 11,000*g* for 20 min to remove insoluble cell debris, and the supernatant was assayed before further purification.

To further purify rLAP II, the supernatant was then filtered through a 0.45-μm filter and applied to a Ni^{2+}-NTA-agarose column (bed volume 40 mL). The column was first washed with buffer (20 mM Tris-HCl, pH 8.0) until the eluant had an absorbance at 280 nm of less than 0.01 and then washed with the same buffer containing 50 mM or 100 mM imidazole, and finally the enzyme was eluted with buffer containing 200 mM imidazole. The enzyme-containing eluant was filtered though a Cross Flow Ultrafiltration Cassette with a molecular weight cutoff of 10 kDa to remove the imidazole. The buffer was simultaneously exchanged for a potassium phosphate buffer (pH 8.0) and concentrated to a certain volume before further use.

Electrophoresis and Protein Assay. The purified enzyme was run on a 10% SDS−PAGE gel according to Laemmli.[21] Before electrophoresis, *E. coli* cells that were collected from 1 mL of culture broth and the purified protein were separately mixed with 5× loading buffer, heated at 100 °C for 5 min, and centrifuged at 14,000*g* for 5 min. The proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Enzyme Assay. rLAP II activity was assayed by monitoring the hydrolysis of L-leucine-*p*-nitroanilide (L-Leu-*p*-NA) according to the method of Kuo et al.[19] with a few modifications in some parameters. The reaction mixture contained 50 mM potassium phosphate buffer (pH 8.0) instead of 50 mM Tris-HCl (pH 8.0), 1 μL of 0.01 M Zn^{2+}, and an appropriate amount of the purified enzyme in a total volume of 100 μL. The mixture was preheated at 55 °C for 3 min, and then 10 μL of 20 mM Leu-*p*-NA was added and incubation continued at the same temperature for 10 min longer. One hundred microliters of absolute alcohol instead of 10% acetic acid was added to terminate the reaction. The absorbance at 405 nm was then measured. One unit (U) of LAP activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitroanilide per minute under the assay conditions. The hydrolytic activity of the purified enzyme against several other *p*-nitroanilide derivatives was also determined according to the above method.

To examine the effect of various pH buffers on rLAP II activity, assays were performed at 55 °C in 50 mM citrate-phosphate buffer (pH 3−8), 50 mM potassium phosphate buffer (pH 6−8), 50 mM HEPES buffer (pH 6−8), 50 mM Tris-HCl buffer (pH 8−9), and 50 mM Gly-NaOH buffer (pH 9−12) and assayed under the above conditions. To examine the effect of temperature on rLAP II, enzymatic activity assays were performed in 50 mM potassium phosphate buffer (pH 8.0) at different temperatures ranging from 10 to 90 °C at 10 °C intervals and assayed under the above conditions.

Circular Dichroism Spectroscopy (CD). CD measurements were carried out with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a cell holder thermostatically controlled by circulating water from a bath. The instrument was controlled by Jasco Spectra Manager software. All measurements were performed under nitrogen flow at room temperature unless otherwise stated. The spectra were recorded over a wavelength range of 190−250 nm using a 1-mm cuvette at a scan speed of 100 nm per min and a response time of 1 s. Photomultiplier absorbance did not exceed 600 V in the analyzed spectral region. Each spectrum was the average of three successive scans. Under the same conditions, the corresponding buffer solution was recorded as a blank and subtracted from the sample spectra. Protein samples (0.2 mg/mL) dissolved in buffers at different pH values (4.0, 6.0, 8.0, and 10.0) or with metal ions were examined. All CD spectra were analyzed by the Yang method using the Spectra Manager software. Thermal denaturation experiments were performed by monitoring the ellipticity at 222 nm. The temperature was increased at a heating rate of 2 °C per minute from 20 to 100 °C.

Hydrolysis of Anchovy Proteins. *Materials.* Anchovies (*Engraulis japonicus*) were purchased from Zhoushan supermarket in Zhejiang, China. Fresh fish were collected after capture and stored in a polyethylene bag at −80 °C until use. Fish were ground to uniformity before use in the present experiments. Flavourzyme 500 MG was obtained from Novozymes China Inc. (Jinan, Baitai).

Production of Protein Hydrolysate by Commercial Enzymes Combined with rLAP II. As anchovy protein extracts exhibit endogenous enzymatic hydrolysis activity, four groups (shown in Table 1) were designed for comparison of the combined effects of rLAP II with endogenous enzymes or other enzymes on the hydrolysis of anchovy proteins. For the last two groups, two steps of hydrolysis were designed: for the third group, the protein was first hydrolyzed with endogenous enzyme for 6 h, then rLAP II was added for further hydrolysis under its optimal conditions; for the fourth group, the

Table 1. Experiment Design and Parameters for Enzymatic Hydrolysis of Anchovy Protein

group	enzyme	temperature	pH
1	endogenous enzyme	55.0 °C	7.0
2	endogenous enzyme + Flavourzyme	50.0 °C	6.5
3	endogenous enzyme + rLAP II	55.0 °C	7.0 for 6 h and then 8.0 for 12 h
4	endogenous enzyme + Flavourzyme + rLAP II	50.0 °C for 6 h and then 55 °C for 12 h	6.5 for 6 h and then 8.0 for 12 h

protein was first hydrolyzed with endogenous enzyme and Flavourzyme for 6 h, and then rLAP II was added for further hydrolysis under its optimal conditions. Reaction parameters for the enzymatic hydrolysis of anchovy proteins are shown in Table 1. The hydrolysis experiments were carried out using the pH-stat method in a 250-mL glass reactor under controlled conditions (pH, temperature, raw material-to-water ratio, and stirring speed). During hydrolysis, the pH and temperature were maintained at the optimal values for the respective enzymes by the addition of 2 N NaOH. A 10-g portion of anchovy protein extract was suspended in 20 mL of deionized water, and enzyme was added to a final concentration of 2000 U of Flavourzyme and 40 U of rLAP II. The reaction was conducted in a thermostatic water bath with a stirring speed of 200 rpm. The hydrolysis time was 18 h, and samples were taken at 2-h intervals. Reactions were terminated by heating the mixture in boiling water for 20 min. The hydrolysate was then centrifuged at 10,000*g* for 30 min, and the supernatant was filtered using a 0.22-μm microfilter in order to remove suspended solids. The supernatant was collected and frozen at −70 °C until further use.

Measurement of the Degree of Hydrolysis (DH). DH was defined as the percentage of free amino groups cleaved from the protein and was calculated as the ratio of α-amino nitrogen to total nitrogen. According to the method of Nilsang et al.,[22] the amino nitrogen content was determined by a formaldehyde titration method. The total nitrogen content was determined by the Kjeldahl method (the conversion factor is 6.25 for loach protein).[23]

Free Amino Acid Analysis. Free amino acid composition was determined by reversed-phase HPLC analysis of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives. The amino acids were treated with AQC to form AQC derivatives, which were then analyzed using a Waters HPLC system (Millipore Ltd.) fitted with a reversed-phase C18 column.

Molecular Weight Distributions. The molecular weight distributions of the hydrolysates were determined using high-performance size-exclusion chromatography (HP-SEC) on a Superdex Peptide HR 10/300 GL column (10 × 300 mm, Amersham Biosciences Co., USA) with a UV detector at 214 and 280 nm. The mobile phase (isocratic elution) was 0.02 M sodium phosphate buffer containing 0.25 M NaCl (pH 7.2), at a flow rate of 0.5 mL per min. A molecular-weight calibration curve was prepared from the average elution volumes of the following standards: cytochrome C (12,500 Da), aprotinin (6,500 Da), vitamin B12 (1,355 Da), Gly-Gly-Gly (189 Da) and Glycine (75 Da) (Sigma Co., USA). UNICORN 5.2 software (Amersham Biosciences Co., USA) was used to analyze the chromatographic data.

Statistical Analysis. All experiments were performed in triplicate. The separation of means was accomplished using one-way analysis of variance (ANOVA) ($P < 0.05$) with the aid of SPSS 13.0 for Windows software. All curves were fitted using Microsoft Office Excel 2003 and Origin 8.0.

RESULTS AND DISCUSSION

Expression and Purification of the Recombinant Enzyme. In this part, much effort was focused on heterogeneous gene expression of rLAP II in *E. coli* BL21 (DE3) for the enhancement of protein quantity (data not shown). To optimize production of rLAP II, IPTG at a final concentration of 0.05 mM and an induction temperature and time of 20 °C and 12 h, respectively, was used in subsequent experiments. Under these conditions, the recombinant protein (rLAP II) was soluble in the supernatant of the cell lysate, which was beneficial for further purification.

rLAP II in the crude extract was further purified by nickel column chromatography. Eluants of 50 mM, 100 mM, and 200 mM imidazole were mixed with loading buffer, and SDS−PAGE analysis was run. As can be seen from the electropherogram, the 200 mM eluant fractions contained most of the rLAP II (Figure 1). The enzymatic activity of the eluant fractions was

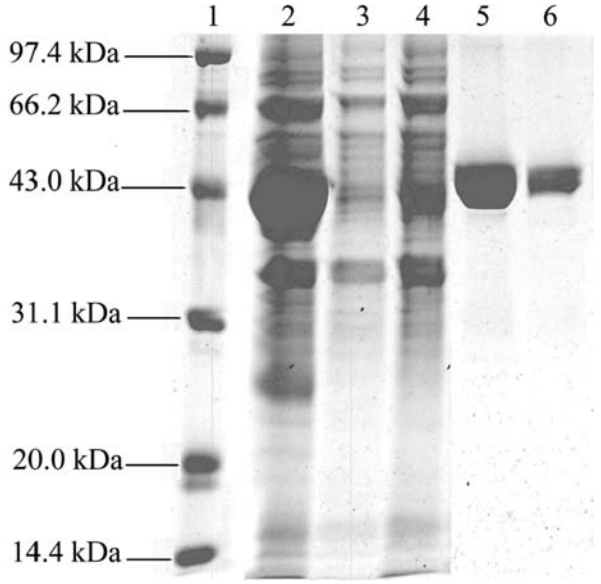

Figure 1. SDS−PAGE analysis of total cell proteins from *E. coli* BL21 (DE3) (harboring pET28a-LAP) and the eluted fractions from nickel-chelate chromatography. Lanes 1, molecular marker; 2, total cell lysate; 3, eluted with washing buffer that contained 50 mM imidazole; 4, eluted with washing buffer that contained 100 mM imidazole; 5, eluted with washing buffer that contained 200 mM imidazole; 6, samples filtered through a Cross Flow Ultrafiltration Cassette with a molecular weight cutoff of 10 kDa.

further confirmed. SDS−PAGE analysis of the purified proteins showed a predominant protein band with an apparent molecular weight of approximately 46 kDa (Figure 1), in close agreement with that of the wild-type enzyme.[18] The eluant fraction containing the enzyme was further filtered through a Cross Flow Ultrafiltration Cassette with a molecular weight cutoff of 10 kDa to remove the imidazole. Finally, rLAP II was successfully expressed and purified before further experimentation. The specific activity of the purified enzyme was 42.5 U/mg protein.

Biochemical Properties and Conformation Changes of rLAP II under Different Conditions. *Effect of pH on the Enzymatic Activity and Secondary Structure of rLAP II.* The enzyme was active within a narrow pH range (8.0 to 9.0), with optimum activity at pH 8.0. However, at pH 8.0, the enzymatic activity also varied in different buffers (50 mM potassium phosphate buffer, 50 mM HEPES buffer, and 50 mM Tris-HCl buffer), with optimum activity in 50 mM potassium phosphate buffer at pH 8.0 (Figure 2A). These results showed that cations or anions in these buffers may have a negative

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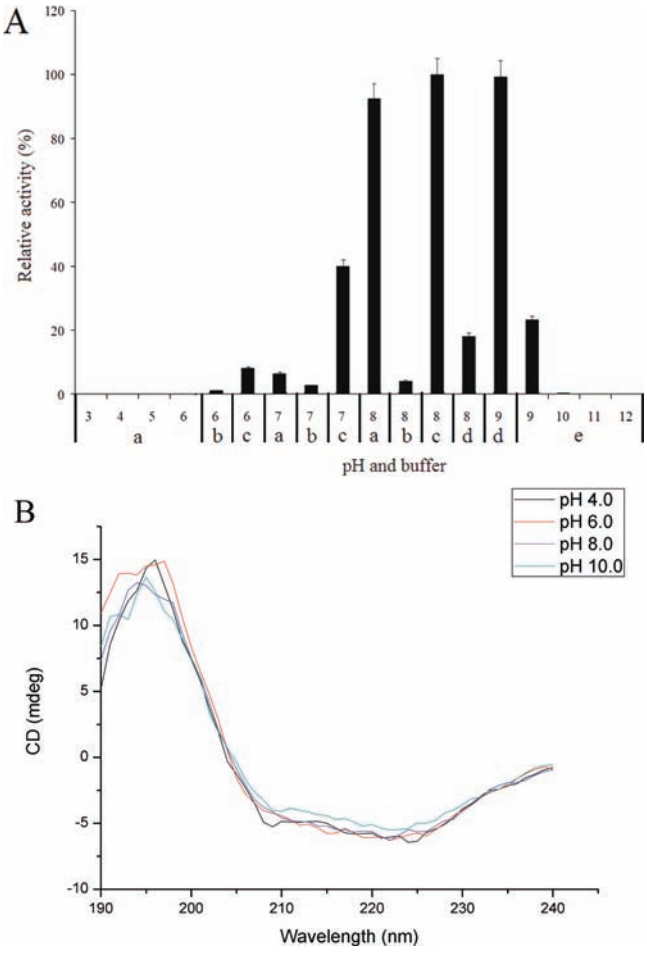

Figure 2. Relative enzymatic activity and circular dichroism of rLAP II under various pH buffers. (A) Relative activity of rLAP II. a: citrate-phosphate buffer. b: HEPES buffer. c: potassium phosphate buffer. d: Tris-HCl buffer. e: glycine-NaOH buffer. Enzymatic activities were measured at various pH values under standard assay conditions. The enzymatic activity of the enzyme in potassium-phosphate buffer (pH 8.0) was taken as 100%. (B) Circular dichroism of rLAP II under various pH buffers.

effect on rLAP II activity by interacting with the active site. However, the details on how these ions could influence the activity of rLAP II still need further research. The enzymatic activity was completely lost when the pH was lower than 6.0 or higher than 10.0 (Figure 2A). Purified rLAP II was stable in the optimal pH buffer. Approximately 80% of LAP activity was retained at its optimal pH of 8.0 for 1 month (data not shown).

As can be seen from the CD results, some modifications were found when rLAP II dissolved in different buffers (Figure 2B). On the basis of the CD spectrum, fractions of different structure were determined. The main backbone conformations of rLAP II at pH 8.0 were α-helix (70.87%), turn (27.30%), and random (1.80%), and no β-sheet was found. However, the α-helical content changed to 65.87% (pH 4.0) ($P < 0.05$) or 76.67% (pH 6.0, 10.0) ($P < 0.05$), indicating that the pH could affect the α-helical regions of the protein molecules and result in enzymatic activity changes. These results could partly explain why rLAP II has no enzymatic activity at lower pH values such as 4.0 and 6.0 or at the higher pH of 10.0. It appears that the α-helical conformation plays an important role in the enzymatic function of rLAP II, with small changes resulting in a dramatic change in enzymatic activity.

Effect of Temperature on the Enzymatic Activity and Secondary Structure of rLAP II. The effect of temperature on the activity of rLAP II was determined in 50 mM potassium phosphate buffer (pH 8.0). The activity was similar from 40 to 60 °C, with an optimum temperature of 55 °C. However, the enzymatic activity declined quickly when the temperature was raised above 60 °C, and increasing the temperature to 70 °C resulted in an almost complete loss of enzymatic activity (Figure 3A).

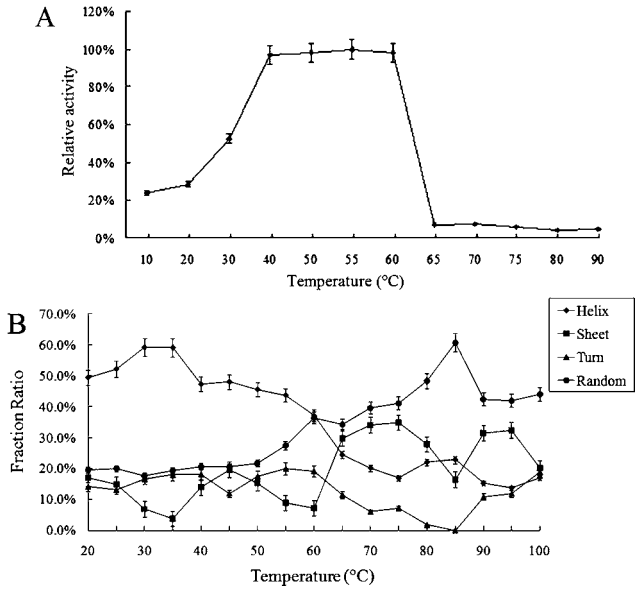

Figure 3. Relative enzymatic activity and variation in different secondary structures of rLAP II under different temperatures. (A) Relative activity of rLAP II at different temperatures. (B) Variation in secondary structures under different temperatures. For detailed methods, see Materials and Methods.

In this study, the effect of temperature on the secondary structure of rLAP II was also examined to try to explain the mechanism from a molecular structure point of view. The CD results are shown in Figure 3B. The α-helical content of rLAP II greatly decreased with increasing temperature, whereas the random structure increased with rising temperature, which means that the structure experienced unfolding and a degenerative process. The α-helical structure of rLAP II was relatively stable within the temperature range of 40 to 55 °C; however, the α-helical structure decreased greatly when the temperature was raised to 60 °C. These results are consistent with the results for enzymatic activity at different temperatures. When the temperature was raised to 60 °C, the secondary structure of rLAP II was greatly changed, causing deactivation of the enzyme.

Effects of Cations and EDTA on the Enzymatic Activity and Secondary Structure of rLAP II. As can be seen from Table 2, among various cations studied here, enzymatic activity was strongly inhibited by Hg^{2+}, and no activity remained at the concentration of 2×10^{-3} M. Inhibition by Hg^{2+} indicates the importance of the indole amino acids for enzyme's function,[24] as has been demonstrated for LAP of *Lactobacillus curvatus* DPC2024.[25] Metallo-aminopeptidases exhibit a broad range of metal-ion dependencies. LAP showed enhanced activity in the presence of several metal ions, like other cobalt-activated metalloenzymes.[25,26] Co^{2+} ions have a strong stimulatory effect

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Table 2. Effects of Various Divalent Cations and Reagents on the Activity of rLAP II

metal ions	concentration (M)	relative activity (%)
none		100
Hg^{2+}	1×10^{-5}	3
	1×10^{-4}	2
	1×10^{-3}	1
	2×10^{-3}	0
Mn^{2+}	1×10^{-4}	156
Cu^{2+}	1×10^{-4}	132
Ni^{2+}	1×10^{-4}	105
Ba^{2+}	1×10^{-4}	124
Mg^{2+}	1×10^{-4}	306
Fe^{2+}	1×10^{-4}	220
Ca^{2+}	1×10^{-4}	141
Zn^{2+}	1×10^{-3}	469
	1×10^{-4}	582
	1×10^{-5}	379
	5×10^{-5}	282
Li^{2+}	1×10^{-4}	316
Fe^{3+}	1×10^{-4}	207
Co^{2+}	1×10^{-4}	2635
EDTA	5×10^{-4}	85

on the catalytic activity of rLAP II (Table 2). All other metal ions tested here (with a rank order of Zn^{2+} > Li^{2+} > Mg^{2+} > Fe^{2+} >Fe^{3+} > Mn^{2+} > Ca^{2+} > Cu^{2+} > Ba^{2+} > Ni^{2+}) had stimulatory effects on the enzymatic activity of rLAP II. LAPs have been characterized as consisting of site 1, which readily exchanges Zn^{2+} for other divalent metal cations including Mn^{2+} and Co^{2+}, and site 2, which binds Zn^{2+} much more strongly and retains Zn^{2+} under conditions that allow the exchange of Zn^{2+} in site 1. In this study, the activation of LAP by Co^{2+} and other metal ions could be attributed to the substitution of Zn^{2+} at site 1 with these metal ions. Indeed, substitution of the site 1 Zn^{2+} of porcine kidney LAP with Mn^{2+} and Co^{2+} has been shown to activate that enzyme by elevating k_{cat}.[27] The activity of EDTA-treated enzyme was slightly reduced at a concentration of 5×10^{-4} M, which was consistent with the results of Kuo et al.[19]

Different conformations of the secondary structure were observed from CD results when various divalent cations were added to the solution (Figure 4A). The secondary structure of rLAP II was greatly modified after Hg^{2+} was added. Compared to the control group with no divalent cations added, a decrease in α-helical content (from 70.87% to 67.27%) and an increase in turn structure (from 27.30% to 30.23%) were observed after Hg^{2+} was added (Figure 4A). Moreover, the proportion of α-helical structure decreased and that of turn structure increased greatly with increasing concentrations of Hg^{2+} (Figure 4A). Hg^{2+} has a negative effect on the enzymatic activity of rLAP II, as previously mentioned. In the present results, Hg^{2+} caused substantial changes in the secondary structure of rLAP II, thereby inducing the complete loss of rLAP II activity. Other divalent cations also had some effect on the secondary structure of rLAP II, which explains why divalent cations had an effect on enzymatic activity. EDTA had little effect on the secondary structure of rLAP II at a concentration of 5×10^{-4} M and only caused a small decrease in enzymatic activity. This may be because the concentration was too low to affect the structure. However, Kuo et al.[19] found that the enzymatic activity was strongly inhibited by EDTA at a concentration of 10 mM. Because of the restriction that the photomultiplier absorbance could not exceed 600 V in the analyzed spectral region, we could not obtain information on the secondary structure of rLAP II at an EDTA concentration of 10 mM.

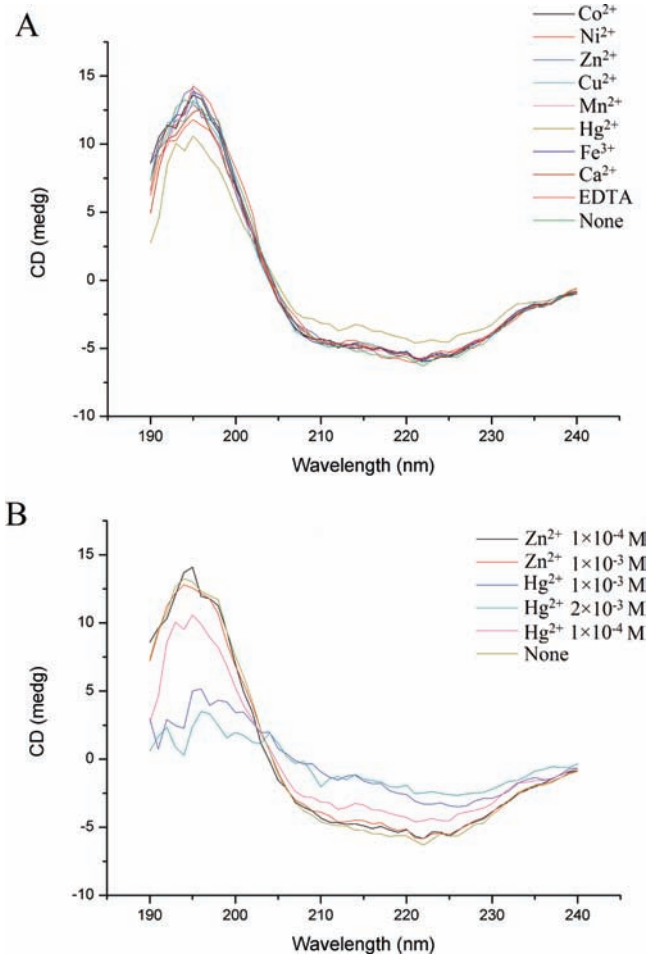

Figure 4. Circular dichroism of rLAP II treated with various concentrations of metal ions or EDTA. rLAP II was dissolved in potassium phosphate buffer (pH 8.0) that contained different concentrations of metal ions or EDTA and measured at room temperatue. (A) The concentration of various divalent cations was 1×10^{-4} M. The concentration of EDTA used in the present study was 5×10^{-4} M. (B) Treated with various concentrations of metal ions.

The secondary structure of rLAP II was also greatly modified after different concentrations of Zn^{2+} were added (Figure 4B). The content of α-helical structure greatly increased (from 70.87% to 75.17%) and the turn structure decreased (from 1.80% to 1.00%) with increasing Zn^{2+} concentrations. Moreover, comparison of samples with two different concentrations of Zn^{2+} (1×10^{-3} M and 1×10^{-4} M) showed that, although they have the same proportion of α-helical structure, the proportions of turn and random structure are different (Figure 4B). The enzymatic activity was higher at 1×10^{-4} M of Zn^{2+} than at 1×10^{-3} M of Zn^{2+}, which means that the turn and random structures also play an important role in the LAP activity of the enzyme.

Substrate Selectivity of rLAP II. Among the *p*-nitroanilide derivatives, rLAP II was most active against Leu-*p*-NA (100%), followed by Met-*p*-NA (36%) and Gly-*p*-NA (19%) (Figure 5). Kuo et al.[19] found that rLAP II was most active against Leu-*p*-NA (100%), followed by Arg-*p*-NA (46.3%), Lys-*p*-NA (37.8%), and Ala-*p*-NA (15.2%). These results together

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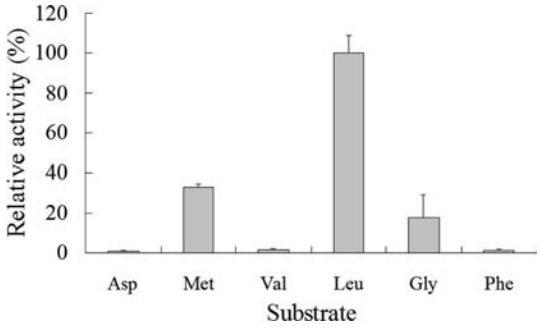

Figure 5. Substrate selectivity of purified rLAP II. Enzymatic activities were measured using various substrates under standard assay conditions.

showed that the enzyme had broad substrate selectivity. However, negligible activity was detected against Asp-*p*-NA, Met-*p*-NA, or Gly-*p*-NA. The preference for Leu-*p*-NA and the optimal values for the reaction pH and temperature indicate that the overexpression system produced an rLAP II functionally identical to the native enzyme.[28]

rLAP II Greatly Promotes the Hydrolysis of Anchovy Proteins. It has been proved that hydrolysates of protein produced by single proteases often display a limited degree of hydrolysis. One enzyme alone cannot achieve a high degree of hydrolysis (DH) within a reasonable period of time.[29,30] A higher degree of hydrolysis can be obtained by using combinations of endo- and exoproteases containing preparations such as Alcalase and Flavourzyme.[31] Accordingly, four groups of experiments were designed in the present study to evaluate the hydrolytic activity of rLAP II and explore possible combinations of proteases for the efficient production of protein hydrolysates of anchovy.

The DH of anchovy was determined, and the results are shown in Figure 6A. The DH of anchovy protein was about 16% after 16 h of autolysis by endogenous enzymes alone (group 1), and it increased to approximately 24% when Flavourzyme was combined with the endogenous enzymes (group 2). For groups 3 and 4, after 6 h of hydrolysis of groups 1 and 2, respectively, rLAP II protease was added, and it was found that the DH of anchovy protein increased rapidly to 27.5% (group 3) and 38.7% (group 4) (Figure 6A). These results indicated that rLAP II protease has good hydrolytic activity, and rLAP II showed positive coordination in the hydrolysis of anchovy protein by comparing the DH value of group 3 with that of group 4.

The molecular weight distribution of the hydrolysates (Figure 6B) showed that all were rich in fractions with lower molecular weights (less than 1000 Da), thus corroborating the higher overall DH observed in the study, and indicated that anchovy proteins were highly degraded by the protease. Quist et al.[8] found that proteins are degraded into higher molecular weight peptides first by endoproteases, then sequential treatment with exoproteases dissociates them into smaller fragments. Flavourzyme is a mixture of enzymes that has both exopeptidase activity and endopeptidase activity. Accordingly, compared with group 1, the molecular weight distribution of peptides of molecular weight >1000 Da was significantly decreased in group 2 ($P < 0.05$). This means that Flavourzyme promoted the hydrolysis process. Moreover, the number of peptides with molecular weight distribution >1000 Da also significantly decreased when rLAP II was added to group 4 ($P <$

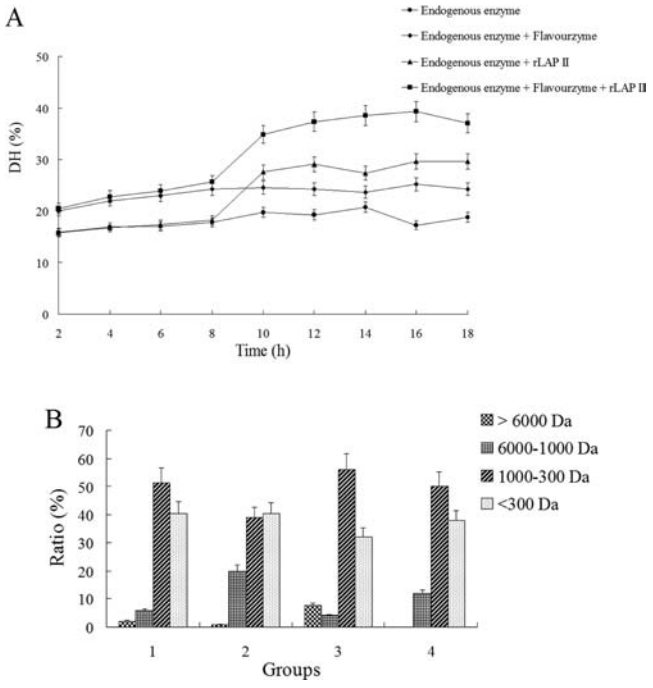

Figure 6. DH and molecular weight distributions (%) of various combinations of proteases in the hydrolysis of anchovy proteins. (A) DH of different groups. (B) Molecular weight distribution (%) of the peptides in the hydrolysates of different groups. For detailed methods, see Materials and Methods.

0.05), compared with the corresponding groups 1 and 2 (Figure 6B). These results indicated that, as an exopeptidase, rLAP II played an important role in the hydrolysis of anchovy proteins. Accordingly, it had great potential to be used for debittering protein hydrolysates with N-terminal hydrophobic amino acids and could be commonly used as clinical nutrition supplements.

The amino acid compositions of different hydrolysates are shown in Table 3. A positively coordinated proteolysis of

Table 3. Free Amino Acid Composition (g/100 mL) in Anchovy Protein Hydrolysate of Different Experimental Groups

	group 1	group 2	group 3	group 4
Asp	139.03	179.62	152.66	226.31
Glu	181.22	203.05	259.45	361.56
Ser	135.09	225.65	159.26	251.6
Gly	60.06	90.83	110.02	184.61
His	115.55	235.00	122.44	232.42
Arg	347.31	398.17	447.37	509.56
Thr	95.54	182.94	145.22	226.62
Ala	173.87	230.44	261.62	352.8
Pro	27.48	45.99	41.94	73.35
Tyr	85.78	104.84	172.65	215.4
Val	138.53	211.30	213.47	295.45
Met	95.27	129.20	123.91	165.53
Cys	5.88	15.40	8.72	15.02
Ile	131.57	197.37	191.03	264.08
Leu	268.26	372.34	383.73	473.08
Phe	137.05	174.54	189.45	232.38
Lys	300.50	362.33	395.58	472.23
total	2437.99	3359.03	3378.52	4552.57

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anchovy proteins, which was catalyzed by a combination of endogenous enzymes, Flavourzyme and rLAP II, seemed to be the most efficient in liberating amino acids, and this was most noticeable in group 4 after 18 h of hydrolysis. This was consistent with the results of DH and molecular weight distribution in Figure 6. rLAP II, as an aminopeptidase, played an important role in generating free amino acids, especially leucine, arginine, and lysine, and showed a broad substrate selectivity as previously mentioned. The essential amino acids, His, Arg, Thr, Tyr, Val, Ile, Leu, and Phe, were observed in the hydrolysates, and all were present at high concentrations. The high level of essential amino acids in the hydrolysates may represent an interesting way to upgrade those hydrolysates for feed in aquaculture or for animal nutrition in general, in addition to being a nitrogen source for microbial growth media. Therefore, anchovy as a quality protein source may have broad applications, providing nutritional additives, debitterness agents, flavor enhancers, functional ingredients, and so on.

In summary, the native secondary structure of rLAP II has a significant impact on its enzymatic activity. Environmental factors could induce conformational alterations in rLAP II and thus influence its activity. rLAP II demonstrated great potential for use in the protein hydrolysis industry, and a combination of endogenous enzymes, Flavourzyme and rLAP II, enhanced the hydrolysis of anchovy proteins and increased the proportions of free amino acids in the hydrolysates. However, the optimized amounts of rLAP II combined with the various amounts of Flavourzyme to promote the production of peptides and amino acids were still unknown from the present results. Therefore, much research still needs to be done to obtain detailed information on the optimized amount of rLAP II for combination by using the corresponding methods (such as orthogonal experiments and response surface methodology). Moreover, anchovies, as an abundant bioresource in the Chinese fishery industry, could be further processed using the hydrolysis of anchovy protein to produce valuable products and result in a profitable business for the protein processing industry.

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