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Leucine Aminopeptidase from Red Sea Bream (*Pagrus major*) Skeletal Muscle: Purification, Characterization, Cellular Location, and Tissue Distribution

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A leucine aminopeptidase was purified for the first time from marine fish red sea bream (*Pagrus major*) skeletal muscle to homogeneity with 4850-fold and a yield of 7.4%. The purification procedure consisted of ammonium sulfate fractionation and chromatographies including DEAE-Sephacel, Sephacryl S-200, hydroxyapatite, and phenyl-Sepharose. The enzyme was approximately 96 kDa as estimated by SDS-PAGE and gel filtration and preferentially hydrolyzed substrate Leu-MCA. The enzymatic activity was optimal at 45 °C and pH 7.5. The K_m and k_{cat} values of the enzyme for Leu-MCA were 1.55 μM and 26.4 S^{-1} at 37 °C, respectively. Activation energy (E_a) of the enzyme was 59.6 kJ M^{-1}. The enzyme was specifically inhibited by metal-chelating agents, and Zn^{2+} and (or) Mn^{2+} seemed to be its metal cofactor(s). In addition, bestatin strongly inhibited its activity, and K_i was 1.44 μM. Using a highly specific polyclonal antibody, the location of enzyme was demonstrated intracellularly and distributed in different tissues.

KEYWORDS: Red sea bream; leucine aminopeptidase; purification; characterization; Western blot

INTRODUCTION

Aminopeptidases (EC 3.4.11.1−15) are a group of exopeptidases with the ability to hydrolyze amino acid residues from the amino terminus of proteins or peptides and classified according to the preference for amino-terminal amino acid of substrates, their location, sensitivity to inhibitors, and requirement of divalent metal ions for their enzymatic activities. These enzymes are considered to have their original substrate specificities depending on their physiological roles and are widely distributed throughout animals, plants, and microorganisms (*1*). Aminopeptidases are found in many subcellular organelles, in the cytoplasm and as a membrane component (*2*). They have medical and biological importance because of their functions in the metabolism of hormones and neurotransmission, cell maturation, and turnover of proteins, including utilization of exogenous proteins as nutrient substances and elimination of nonfunctional proteins (*3, 4*). In muscle tissues, aminopeptidases are involved in the generation of free amino acids in meat and meat products and consequently are important in the improvement of the taste of meats (*5*). Several types of aminopeptidases have been identified in muscle, including leucyl aminopeptidase and pyroglutamyl aminopeptidase from human skeletal muscle (*6*), methionyl aminopeptidase from porcine muscle, and aminopeptidase H from bovine and chicken muscle (*5, 7, 8*). Aminopeptidase D was recently purified from bovine skeletal muscle (*9*). It has been believed that aminopeptidases together with other endogenous proteinases play important roles in muscle autolysis during post-mortem storage (*10*−*12*).

Leucine aminopeptidases (LAPs) are often viewed as cell maintenance enzymes with critical roles in the turnover of peptides and as the final step in protein degradation (*13*). In mammals, LAPs are involved in peptide processing for MHC I antigen presentation, production of bioactive peptides (oxytocin, wasopressin, enkephalins), and vesicle trafficking to the plasma membrane. LAP from mammalian tissues has also been extensively studied because of its association with a number of pathological conditions such as eye lens aging and cataract formation (*14*). The roles of LAP in plants concerning defense, membrane transport of auxin receptors, and meiosis have also been implicated (*15*). In microbes, LAPs serve as transcriptional repressors to control pyrimidine, alginate, and cholera toxin biosynthesis, as well as mediate site-specific recombination events in plasmids and phages (*13*). More recently, the production of LAP from microorganisms by fermentation has been

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paid considerable attention due to its regulating the levels of bitter peptides and improving flavor in the food industry (*16*−*18*).

In contrast to aminopeptidases from mammals, plants, parasites, and microorganisms, there is a limited knowledge about aminopeptidases from fish. Until now, only an alanyl aminopeptidase from Alaska pollack roe and a methionine aminopeptidase from tuna pyloric ceca have been purified (*19, 20*), and most recently, we reported the purification of a LAP from the freshwater fish common carp (*21*), whereas LAP from marine fish has never been reported. On the other hand, fish sauce is a popular Chinese traditional condiment, which mainly uses marine fish as raw materials. Thus, understanding the characteristics of endogenous proteinases in fish muscle will surely be beneficial for the production of such products as fish sauce. In this paper, we described the purification and characterization of a LAP from the skeletal muscle of red sea bream. The cellular location and tissue distribution of LAP were also investigated using a highly specific rabbit antiserum. Our present data considerably expanded the thus far rather limited knowledge of aminopeptidases in marine fish.

MATERIALS AND METHODS

Fish. Cultured red sea bream (*Pagrus major*) (body weight of 600−700 g) was purchased alive from a fish market of Jimei, Xiamen. The fish was sacrificed instantly. After decapitation and evisceration, skeletal muscle was immediately used for experiment.

Chemicals. DEAE-Sephacel, Sephacryl S-200 HR, and phenyl-Sepharose 6-Fast Flow were purchased from Amersham Biosciences (Uppsala, Sweden). Econo-Pac CHT-II Cartridge hydroxyapatite column, molecular weight calibration marker for gel filtration, and dithiothreitol (DTT) were from Bio-Rad (Hercules, CA). L-Arginine-4-methylcoumaryl-7-amide hydrochloride (Arg-MCA), *tert*-butyloxycarbonyl-Phe-Ser-Arg-4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA), *tert*-butyloxycarbonyl-Leu-Arg-Arg-4-methyl-coumaryl-7-amide (Boc-Leu-Arg-Arg-MCA), and *tert*-butyloxycarbonyl-Leu-Lys-Arg-4-methyl-coumaryl-7-amide (Boc-Leu-Lys-Arg-MCA) were obtained from Peptide Institute (Osaka, Japan). Other fluorogenic substrates (MCA substrates), bestatin hydrochloride, bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline monohydrate, and ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were products of Sigma (St. Louis, MO). L-3-Carboxy-*trans*-2,3-epoxypropionyl-L-leucin-4-guanidinobutylamide (E-64) was obtained from Amresco (Solon, OH). Pepstatin was from Roche (Mannheim, Germany). Both peroxidase-conjugated and FITC-conjugated goat anti-rabbit immunoglobulin G were purchased from Pierce (Rockford, IL). Protein marker for SDS-PAGE was from Fermentas (Vilnius, Lithuania). Other reagents were all of analytical grade.

Assay of Aminopeptidase Activity. Routinely, aminopeptidase activity was measured using Leu-MCA as substrate according to the method of Liu et al. (*21*) with some modification. Appropriately diluted enzyme (10 μL) was added to 940 μL of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.02% NaN_3 (buffer A). Fifty microliters of 10 μM fluorogenic substrate was added to initiate the reaction. The reaction was performed at 37 °C for 10 min and stopped by the addition of 1.5 mL of stopping agent (methyl alcohol/*n*-butyl alcohol/distilled water = 35:30:35, v/v/v). Enzymatic activity was detected by measuring the fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm on a fluorescence spectrophotometer (FP-6200, Jasco, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nmol of AMC per minute. Proteolytic activity assays in each case were performed in duplicate, and variation between duplicate samples was always >5%. The mean values were used.

Purification of Aminopeptidase. All procedures were performed at 4 °C. About 380 g of minced fish muscle was homogenized in 3-fold of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT, 0.02% NaN_3 (buffer A) using a homogenizer (Kinematica, PT-2100, Littau-Lucerne, Switzerland) and centrifuged at 10000*g* for 15 min in a centrifuge (Avanti J-25, Beckman Coulter, Fullerton, CA). The supernatant was fractionated with ammonium sulfate, and a 40−60% $(NH_4)_2SO_4$ precipitate was dissolved in a minimum volume of buffer A and dialyzed overnight against the same buffer. The dialyzed solution was subsequently applied to a DEAE-Sephacel column (2.5 × 27 cm) equilibrated with buffer A. The bound proteins were eluted at 1.0 mL/min with a 0−0.5 M linear NaCl gradient in buffer A, and fractions of 5 mL were collected. Active fractions were pooled and concentrated by ultrafiltration with a membrane of YM-30 (Millipore, Bedford, MA) and then applied to a gel filtration column of Sephacryl S-200 HR (1.5 × 98 cm) equilibrated with buffer A containing 0.2 M NaCl at a flow rate of 0.4 mL/min. Active fractions were pooled and dialyzed against buffer A and subsequently applied to a 5 mL Econo-Pac CHT-II Cartridge hydroxyapatite column pre-equilibrated with buffer A. Unbound proteins were pooled, and ammonium sulfate was added to 1 M and applied to a phenyl-Sepharose 6-Fast Flow column (0.8 × 4 cm) pre-equilibrated with buffer A containing 1 M ammonium sulfate. The proteins retained were eluted at a flow rate of 0.4 mL/min using a linear decreasing gradient of ammonium sulfate from 1 to 0 M in buffer A in a total volume of 80 mL. Active fractions were further collected and dialyzed against buffer A and used for enzymatic characterization or as antigen for polyclonal antibody preparation.

Protein Concentration Determination. Protein concentration was determined by measuring the absorbance at 280 nm of the sample solution on column chromatography or with the method of Lowry (*22*) with bovine serum albumin as standard.

Estimation of Molecular Mass. The molecular mass of the purified enzyme was estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; the gel was stained with Coomassie Brilliant Blue R-250.

A gel filtration performance was also carried out to estimate the molecular mass of the native enzyme on a Sephacryl S-200 column (1.5 × 98 cm) using thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1350 Da) as standards.

Effects of Temperature and pH. Optimal temperature was measured in buffer A at different temperatures (10−65 °C). Thermal stability was determined with the purified enzyme in buffer A at various temperatures for 30 min and then cooled in an ice bath. The remaining activity was assayed by the method as described above.

The optimal pH of the enzyme activity was determined at 37 °C using 50 mM concentrations of the following buffers: sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−7.0), Tris-HCl (pH 7.5−8.5), and Na_2CO_3−$NaHCO_3$ (pH 9.0−10.0). pH stability was determined with the purified enzyme in the above buffers incubated at 4 °C for 48 h, and the remaining activities were assayed at 37 °C in buffer A according to the method as described above. All analyses were carried out in triplicate, and the results are shown as average values ± standard deviation (95% confidence levels).

Effect of Protease Inhibitors and Metal Ions. To investigate the effects of different proteinase inhibitors on the enzyme, purified enzyme was preincubated with corresponding inhibitors at different final concentrations for 30 min at room temperature in buffer A, and the remaining activity was determined under standard assay conditions. Control tests were performed in the absence of inhibitor.

The effect of divalent metal ions on the enzyme was examined at final concentrations of 0.1 and 1 mM. Control tests were performed in the absence of metal ions. To identify the enzyme as a metallopeptidase, purified enzyme was further dialyzed extensively against 1 mM EDTA in buffer A, and then the effect of divalent metal ions on the enzymatic activity under standard assay conditions was detected. All analyses were carried out in triplicate, and the results are shown as average values ± standard deviation (95% confidence levels).

Determination of Kinetic Parameters. The K_m and k_{cat} values for the reaction of the enzyme with Leu-MCA and Arg-MCA as substrates were estimated from two Lineweaver−Burk plots of $1/v$ against 1/[S] over the concentration of fluorogenic substrate range of 0.25−20 μM at 37 °C. The thermodynamic parameters of activation for the enzyme

were calculated by Arrhenius plot using k_{cat} values of four different temperatures from 27−42 °C with Leu-MCA as substrate.

The inhibition type of bestatin was determined on the basis of Lineweaver−Burk plots. Briefly, the purified enzyme was preincubated with four different concentrations of bestatin from 1 to 8 μM followed by reaction with Leu-MCA in five different concentrations from 1 to 20 μM, respectively. The K_i value was calculated according to the method of Cristofoletti (*23*).

Preparation of Polyclonal Antibody and Western Blot Analysis. To obtain polyclonal antibody against the enzyme, purified LAP was adjusted to the concentration of 200 μg/mL and then emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, USA) as antigen. At initial immunization, an adult female rabbit was injected subcutaneously at several sites on the back with 100 μg of antigen in a total volume of 1 mL. At 2, 4, and 5 weeks later, the rabbit received three booster injections intraperitoneally with the same amount of antigen as the initial immunization except that it was emulsified with incomplete Freund's adjuvant (Sigma-Aldrich, USA). Five days later, the rabbit was bled from the retro-orbital plexus to obtain antiserum.

Western blot was performed as described by Towbin (*24*). Briefly, aminopeptidase was electrophoresed in 10% sodium dodecyl sulfate−polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 60 min and then incubated with the rabbit antiserum (1:1000 dilution) for 90 min. After extensive washing with Tris-buffered saline Tween 20 (TBST), blots were then revealed with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2000 dilution; Pierce) using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a substrate.

Tissue Distribution. Different fish tissues, including brain, liver, kidney, stomach, gut, pyloric ceca, gill, and skeletal muscle, were collected and homogenized with buffer A and prepared at approximately the same protein concentration of 2 mg/mL and separated by 10% SDS-PAGE. After electrophoresis, the gel was electrophoretically transferred to a nitrocellulose membrane. Western blot was detected as described above.

Immunohistochemical Analysis. Immunohistochemical analysis of the cellular location of LAP was measured according to the method of Tassy et al. (*25*) with some modification. Briefly, skeletal muscle strips (3 mm × 10 mm) from fresh cuts of red sea bream perpendicular to the fiber axis were immersed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 60 min at room temperature. Samples were then bathed in 30% sucrose in PBS buffer to equilibrium. Thick transverse sections of about 10 μm were cut with a cryostat (Reichert-Jung Frigocut 2800, Heidelberg, Germany) and treated with BSA for 30 min followed by three 5 min successive washings in PBS. The sections were then incubated with the rabbit anti-red sea bream LAP polyclonal antibody (diluted to 1/500 in PBS) for 60 min at room temperature. After three 5 min successive washings in PBS, the sections were treated with the FITC-conjugated goat anti-rabbit immunoglobulin G (diluted to 1/500 in PBS) for 60 min at room temperature, followed by three 5 min successive washings in PBS, and then mounted in glycerol on glass plates. Sections were then examined using a Leica 2E light microscope (Leica, Heidelberg, Germany). Control test was performed using nonimmunized rabbit serum instead of the polyclonal antibody.

RESULTS

Purification of the Aminopeptidase. In the present study, an aminopeptidase from the red sea bream skeletal muscle was purified to homogeneity through five purification procedures. The chromatographic profile on DEAE-Sephacel showed that a single enzymatic active peak was eluted from a linear gradient of 0−0.5 M NaCl in buffer A (**Figure 1A**), whereas most contaminating proteins were removed. After gel filtration chromatography on a Sephacryl S-200 column, the active fraction pool was dialyzed and loaded onto an Econo-Pac CHT-II Cartridge hydroxyapatite column. The unbound protein pool containing most aminopeptidase activity was applied to a phenyl-Sepharose hydrophobic interaction column, and the aminopeptidase was finally purified to homogeneity (**Figure 1C**).

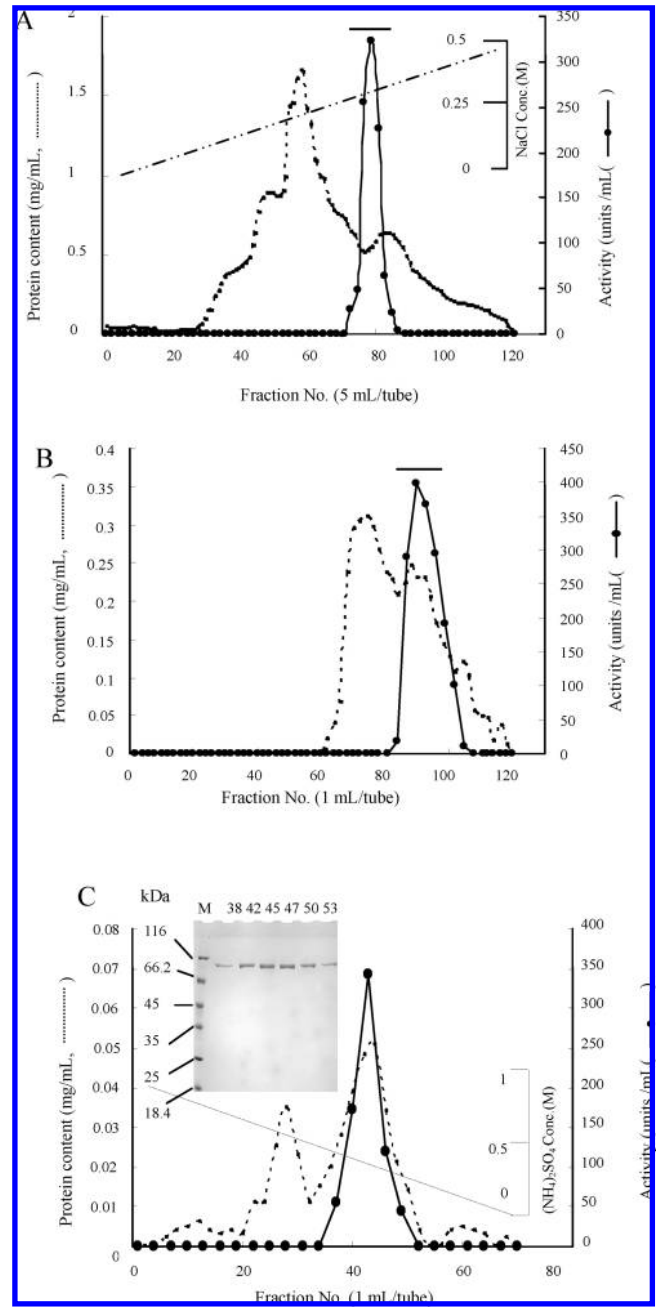

Figure 1. Column chromatography purification of leucine aminopeptidase from red sea bream skeletal muscle: (**A**) DEAE-Sephacel chromatography; (**B**) Sephacryl S-200 chromatography; (**C**) phenyl-Sepharose chromatography. Protein content is represented by a dashed line; Leu-MCA hydrolyzing activity is represented by solid circles.

A summary of the purification is shown in **Table 1**. About 0.29 mg of aminopeptidase was obtained from 380 g of red sea bream skeletal muscle; the overall recovery was 7.4% with a 4850-fold increase in specific activity. To identify the purification efficacy, fractions obtained from each purification step were analyzed by SDS-PAGE. As shown in **Figure 2A**, the molecular mass of aminopeptidase was about 96 kDa on SDS-PAGE, which is in accordance with the gel filtration result estimated using the Sephacryl S-200 column (**Figure 2B**), suggesting the enzyme is a monomer.

Table 1. Summary of Purification of the Aminopeptidase from Red Sea Bream Skeletal Muscle

purification step	total protein (mg)	total activity (units)	specific activity (units mg^{-1})	yield (%)	purification (fold)
cell extract	19073	15640	0.8	100	1
$(NH_4)_2SO_4$ precipitation	2600.6	5643.6	2.2	36.1	2.7
DEAE-Sephacel	9.6	4928	514.9	31.5	628
Sephacryl S-200	2.2	1925.2	887.2	12.3	1082
hydroxyapatite	0.6	1613.9	2882	10.3	3514.6
phenyl-Sepharose	0.29	1153.4	3977.4	7.4	4850.5

Effects of Temperature and pH. As shown in **Figure 3A**, the enzyme revealed an optimum temperature of 45 °C and the activity obviously decreased above 50 °C. It is noteworthy that the enzyme retained 10% of its optimal activity even at 10 °C. A thermostability study revealed that the LAP activity decreased slightly after incubation below 30 °C, but rapidly above 40 °C (**Figure 3B**). The enzyme completely lost its activity upon freezing and thawing, whereas >90% of the enzyme activity remained even after 6 months of storage when the enzyme was kept in 10% sucrose at −80 °C. The pH optimum of the LAP was 7.5 (**Figure 4A**), and the enzymatic activity was negligible below pH 5.5 and above 8.5. A pH stability study showed

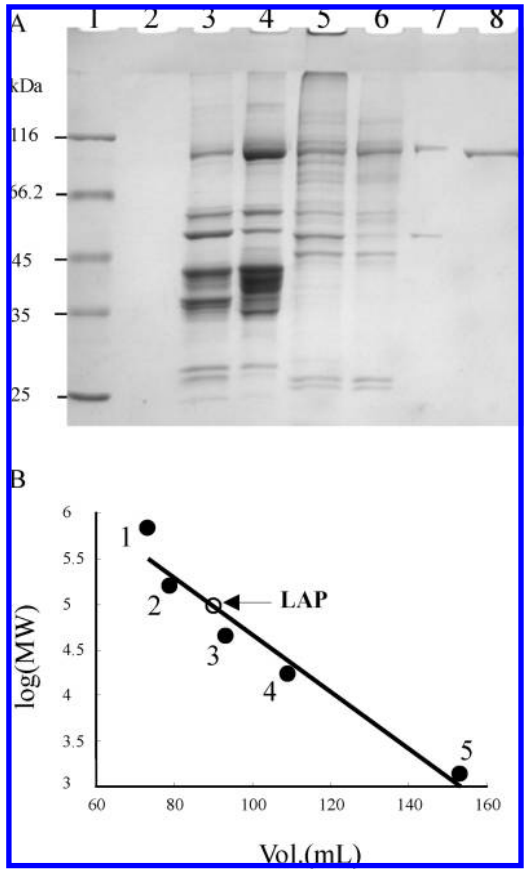

Figure 2. SDS-PAGE of red sea bream LAP and estimation of molecular mass by Sephacryl S-200: (**A**) LAP at each purification stage was subjected to electrophoresis on a 10% gel followed by Coomassie Brilliant Blue staining (lanes: 1, molecular weight marker; 2, blank; 3, crude extract; 4, 40−60% ammonium sulfate precipitation; 5, DEAE-Sephacel fraction; 6, Sephacryl S-200 fraction; 7, Econo-Pac CHT-II Cartridge hydroxyapatite fraction; 8, phenyl-Sepharose fraction); (**B**) Sephacryl S-200 gel filtration estimation of molecular mass [1, thyroglobulin (670 kDa); 2, bovine γ-globulin (158 kDa); 3, chicken ovalbumin (44 kDa); 4, equine myoglobin (17 kDa); 5, vitamin B_{12} (1350 Da); arrowhead indicates the elution position (open circle) of the LAP].

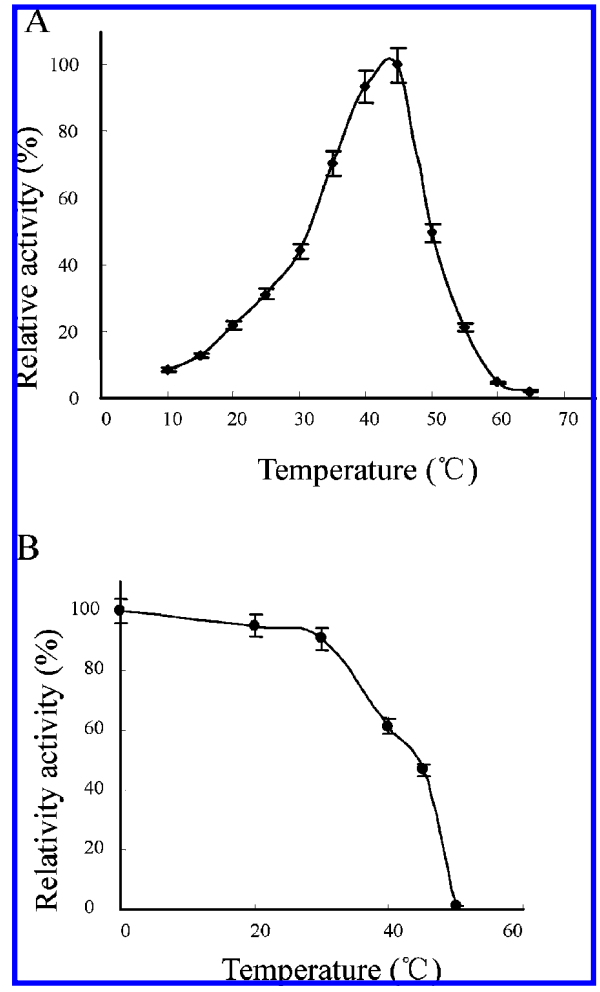

Figure 3. Optimal temperature (**A**) and thermal stability (**B**) of red sea bream LAP: (**A**) LAP activity was assayed with 0.5 μM Leu-MCA as substrate in buffer A for 10 min under different temperatures from 10−65 °C; (**B**) purified LAP was incubated at various temperatures for 30 min. The remaining activity was assayed.

moderate activity loss after incubation below pH 6.0, but rapid loss after incubation above pH 7.5 for 48 h (**Figure 4B**). These data suggested that the LAP is a neutral or slightly alkaline aminopeptidase and that the functional pH value is in a narrow range.

Substrate Specificity. To better define the substrate specificity of the purified enzyme, several fluorogenic substrates have been examined. For the purpose of comparison, all of the results are listed in **Table 2**. The enzyme rapidly hydrolyzed Leu-MCA and Arg-MCA and considerably hydrolyzed other substrates such as Ala-MCA and Tyr-MCA, although with relatively low hydrolysis rates of 20.5 and 7.4%, respectively. However, it did not reveal any endoproteinase activity toward N-terminal-blocked substrates including Boc-Phe-Ser-Arg-MCA, Boc-Leu-Lys-Arg-MCA, and Boc-Leu-Arg-Arg-MCA. The enzyme also did not exhibit apparent proteolytic activity toward protein substrates such as bovine serum albumin and myofibrillar proteins, including myosin heavy chain, actin, and tropomyosin (data not shown). On the basis of substrate specificity, the enzyme was regarded as a leucine aminopeptidase (LAP).

Effect of Protease Inhibitors and Metal Ions. Several proteinase inhibitors were used in the present study to confirm the property of the enzyme. As shown in **Table 3A**, the enzymatic activity was strongly inhibited by EDTA, EGTA, bestatin, 1,10-phenanthroline, and E-64. In contrast, inhibitors

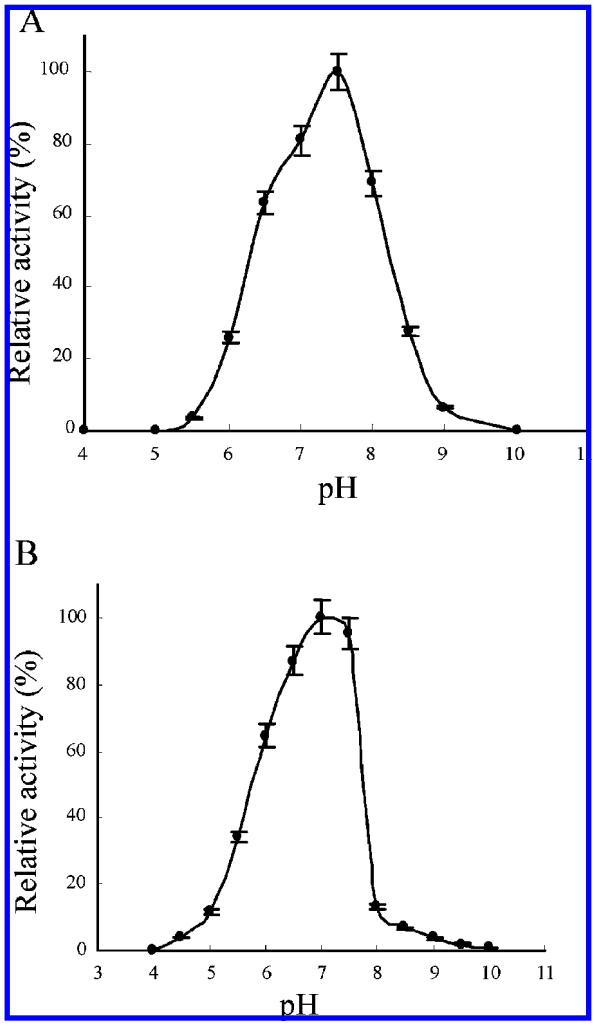

Figure 4. Optimal pH (**A**) and pH stability (**B**) of red sea bream LAP: (**A**) LAP activity was assayed with 0.5 μM of Leu-MCA as substrate in different pH buffers for 10 min at 37 °C; (**B**) purified enzyme in different pH buffers was incubated at 4 °C for 48 h. The remaining activity was assayed at 37 °C in buffer A.

Table 2. Substrate Specificity of Red Sea Bream LAP

substrate	relative activity (%)	substrate	relative activity (%)
Leu-MCA	100	Val-MCA	0
Arg-MCA	70	Gly-MCA	0
Ala-MCA	20.5	Boc-Phe-Ser-Arg-MCA	0
Tyr-MCA	7.4	Boc-Leu-Lys-Arg-MCA	0
Pro-MCA	0	Boc-Leu-Arg-Arg-MCA	0

to serine proteinase (PMSF) and asparatic proteinase (pepstatin) did not show much inhibitory effects. The effect of divalent metal ions is shown in **Table 3B**. At the concentration of 1 mM, the enzyme was strongly inactivated by metal ions of Cu^{2+}, Zn^{2+}, and Cd^{2+} and partially inactivated by Fe^{2+}. Cationic ions Ca^{2+}, Mn^{2+}, and Ba^{2+} showed slight inhibitory effect at 1 mM, but slight activation at 0.1 mM. In contrast, Mg^{2+} slightly activated the enzymatic activity at concentrations from 0.1 to 1 mM. The enzyme lost 83% of its activity when it was dialyzed extensively against 1 mM EDTA in buffer A, whereas the loss could be restored by Zn^{2+} to 97% at 0.05 mM and to 81% at 0.1 mM of its original activity. The recovery of enzymatic activity by Mn^{2+} reached 94% at 0.5 mM and 79% at 1 mM.

Table 3. Effect of Chemicals and Protease Inhibitors (**A**) and Metal Ions (**B**) on LAP Activity

inhibitor	concentration (mM)	residual activity (%)
(**A**) Effect of Chemicals and Protease Inhibitors		
control	0	100
EDTA	5	7.9
EGTA	5	25.4
1,10-phenanthroline	0.2	31.1
bestatin	0.01	22.3
E-64	0.015	40.4
pepstatin	0.15	83.4
PMSF	1	94.9
(**B**) Effect of Metal Ions		
control	0	100
$MgCl_2$	0.1	109.5
	1	108.3
$BaCl_2$	0.1	106.1
	1	97.3
$MnCl_2$	0.1	108.9
	1	90.1
$CaCl_2$	0.1	104.7
	1	86.8
$FeSO_4$	0.1	107.3
	1	53.9
$CdCl_2$	0.1	63.4
	1	38.2
$ZnSO_4$	0.1	55.8
	1	35.1
$CuSO_4$	0.1	13.8
	1	2.3

On the other hand, the enzymatic activity recovery by Ca^{2+} and Mg^{2+} was lower than that by Zn^{2+} and Mn^{2+}; the former reached 46% and the latter, 26%, at 1 mM.

Kinetic Parameters. The Lineweaver−Burk plots of the enzyme using Leu-MCA and Arg-MCA as substrates are shown in **Figure 5A**. The K_m values for Leu-MCA and Arg-MCA both were 1.55 μM. On the basis of the molecular mass of the LAP 96 kDa, the k_{cat} values for Leu-MCA and Arg-MCA were 26.4 and 17.3 s^{-1}, respectively.

An Arrhenius plot of k_{cat} versus $1/T$ was linear in the temperature range of 27−42 °C (**Figure 5B**). From the slope of the line, the activation energy (E_a) of the LAP was calculated to be 59.6 kJ M^{-1}, which was higher than that of *Acyrthosiphon pisum* aminopeptidase, E_a = 42.2 kJ M^{-1} (*23*).

As shown in **Figure 6**, bestatin was a competitive and specific inhibitor of the LAP and its K_i was 1.44 μM.

Preparation of Polyclonal Antibody and Tissue Distribution of LAP. Western blot analysis showed that the immunized rabbit raised specific high-titer polyclonal antibody against the LAP, as a clear band corresponding to the size of LAP was detected, whereas no band was observable on the control test (**Figure 7A**).

Tissue distribution of the LAP was also assessed using Western blot. According to the results presented in **Figure 7B**, all samples, including skeletal muscle, brain, liver, kidney, gut, stomach, pyloric ceca, and gill, generated only a specific band corresponding to the size of LAP, suggesting LAP is widely distributed in the fish body. In addition, with respect to the intensity of the band and the enzymatic activity hydrolyzing Leu-MCA substrate (data not shown), LAP was more abundant in brain and kidney than in other tissues.

Immunolocalization of LAP. Immunohistochemical analysis of LAP was performed on transverse sections of freshly excised red sea bream skeletal muscle using the polyclonal rabbit antiserum described above. As shown in **Figure 8**, compared with control where no green fluorescence was detected (**Figure**

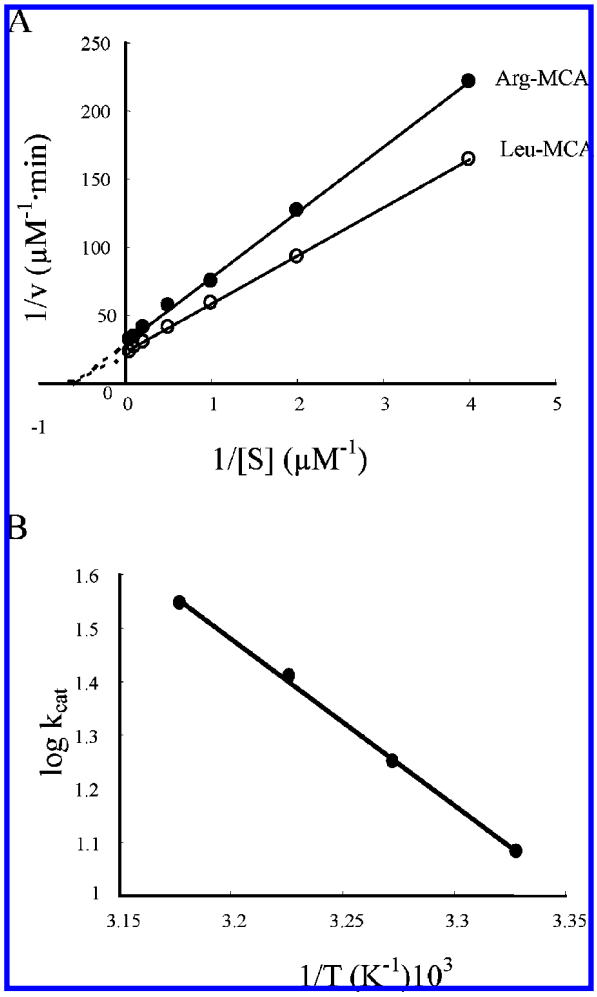

Figure 5. Lineweaver−Burk plots (**A**) and Arrhenius plot of LAP red sea bream (**B**). The K_m value was 1.55 μM, and the k_{cat} values were 26.4 and 17.3 s^{-1} for Leu-MCA and Arg-MCA, respectively. Activation energy was determined as E_a = 59.6 kJ M^{-1}.

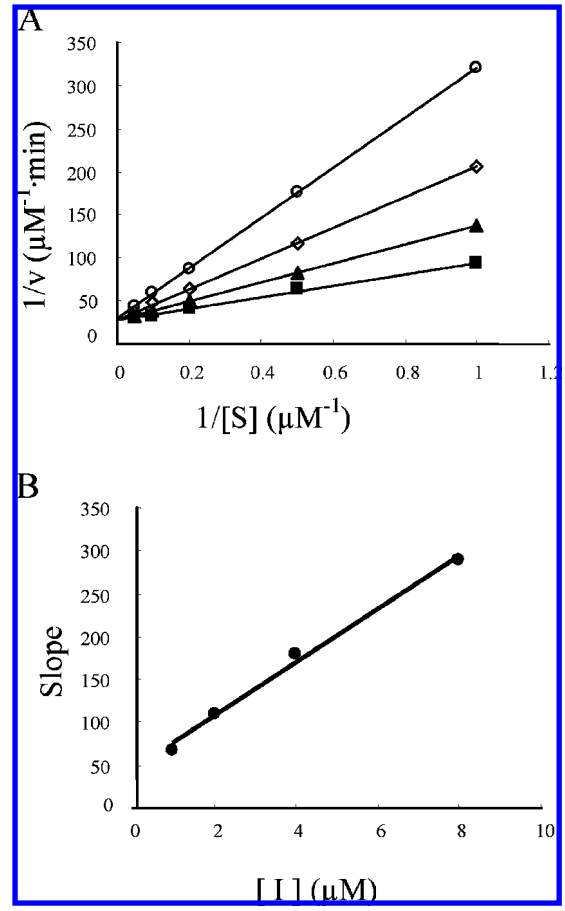

Figure 6. Inhibition of red sea bream LAP by bestatin: (**A**) Lineweaver−Burk plots of LAP-hydrolyzing activity in the presence of different concentrations of bestatin; (**B**) replots of slopes calculated from Lineweaver−Burk plots against the concentration of bestatin.

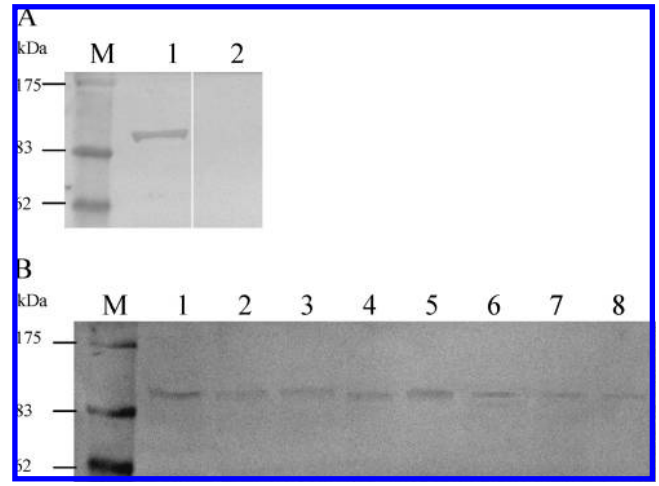

Figure 7. Western blot analysis of the distribution of LAP in different red sea bream tissues: (**A**) lane M, prestained protein marker; lane 1, purified LAP react with rabbit anti-LAP serum; lane 2, purified LAP react with normal rabbit serum; (**B**) tissue distribution of LAP as detected by Western blot using rabbit anti-LAP serum (lane M, prestained protein marker; lane 1, brain; lane 2, stomach; lane 3, pyloric ceca; lane 4, liver; lane 5, kidney; lane 6, skeletal muscle; lane 7, gill; lane 8, gut).

8A), green fluorescence is highly concentrated as a punctate pattern in the cytoplasm (**Figure 8B**), indicating that LAP mainly exists in intracellular vesicles.

DISCUSSION

In the present work, we developed a protocol enabling the purification, to homogeneity, of a LAP from marine fish red sea bream skeletal muscle by ammonium sulfate fractionation and column chromatographies including DEAE-Sephacel, Sephacryl S-200, Econo-Pac CHT-II Cartridge hydroxyapatite, and phenyl-Sepharose. The procedure was developed from the purification of freshwater fish common carp LAP (*21*). On the Econo-Pac CHT-II Cartridge hydroxyapatite chromatography unbound protein was pooled for subsequent chromatography, which was apparently different from the purification of common carp LAP in which bound protein was used for subsequent phenyl-Sepharose chromatography (*21*). This may arise from the different experimental conditions employed in each case or from properties of these enzymes. On average, about 0.29 mg of highly purified LAP was obtained from 380 g of fresh red sea bream skeletal muscle. The LAP revealed an apparent molecular mass of 96 kDa by both SDS-PAGE and gel filtration, indicating that the enzyme exists in a monomeric form. This molecular mass is similar to those of aminopeptidases from common carp (105 kDa) (*21*), pig kidney (94/98 kDa) (*26*), rat brain (110 kDa) (*3*), and Alaska pollack roe (105−125 kDa) (*19*). It is smaller than an aminopeptidase from tuna pyloric ceca (150 kDa) (*20*) and many LAPs from mammals and plants as these enzymes are hexamers composed of about 60 kDa subunits (*6, 13, 15*). However, it is higher than those from

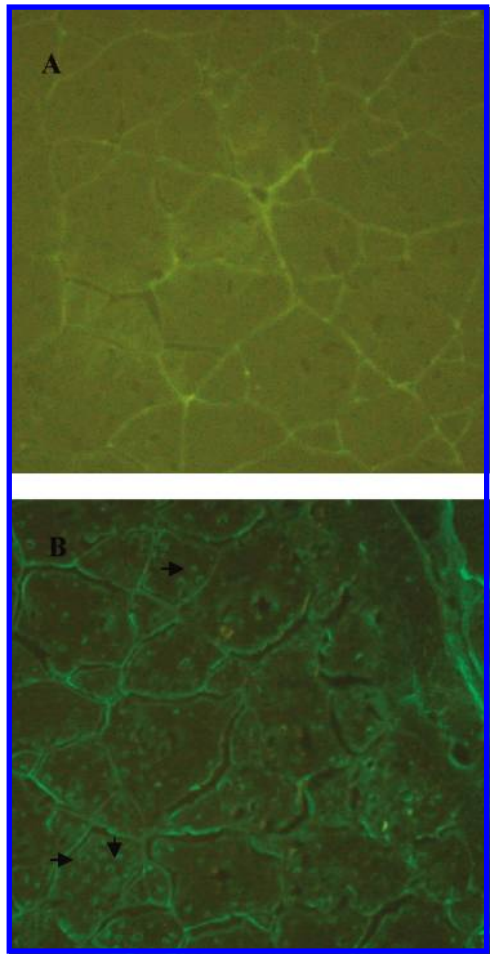

Figure 8. Immunohistochemical localization of LAP in red sea bream skeletal muscle cell: (**A**) control sample incubated with normal rabbit serum; (**B**) sample treated with the specific rabbit antiserum of the LAP. Transverse sections prepared from skeletal muscle were incubated with specific rabbit antiserum of the LAP or normal rabbit serum revealed by FITC-conjugated goat anti-rabbit IgG. Arrowheads indicate intracellular vesicles of muscle cell stained by green fluorescence. (Magnification, ×100).

Bacillus sp. N2 (58 kDa) (*27*), *Aspergillus sojae* (37 kDa) (*16*), and scallop (61 kDa) (*28*).

The LAP revealed an optimum temperature of 45 °C and an optimum pH 7.5 using Leu-MCA as substrate (**Figures 3** and **4**). The LAP retained very low activity both below pH 5.5 and above pH 8.5. This temperature- and pH-dependent characteristic may be ascribed to the accommodation temperature of red sea bream. Accordingly, it can be presumed that LAP may play an active role not only in vivo toward physiological substrates but also in collaboration with other proteinases, such as cathepsins that function in further degradation of peptides during the period of post-mortem (*29*).

Unlike mammalian animal meats in which post-mortem aging is necessary for flavor enhancement, post-mortem tenderization in fish muscle is one of the most unfavorable quality changes (*29*). Therefore, extensive biochemical characterizations of proteinases involved in this process are essential to identify potential quality indicators or to postpone muscle protein degradation during cold storage. During the post-mortem tenderization of animal meat, various endopeptidases are involved, including lysosomal and nonlysosomal proteinases. Although the physiological role of LAP in fish muscle remains unclear, during post-mortem tenderization of fish muscle, like other aminopeptidases (*8*), LAP may be also responsible for the hydrolysis of peptides produced by the processing of endopeptidases to free amino acids when the pH of muscle returns to neutral by the release of amine products.

Substrate specificity of the enzyme revealed that the hydrolysis rates were strikingly affected by amino acid residues of the substrates. The hydrolysis rate was highest to Leu-MCA, secondarily high to Arg-MCA, and relatively lower to Ala-MCA and Tyr-MCA, whereas the enzyme did not catalyze Val-MCA, Gly-MCA and substrates for endopeptidases such as Boc-Phe-Ser-Arg-MCA, Boc-Leu-Lys-Arg-MCA, and Boc-Leu-Arg-Arg-MCA, confirming that it is an exopeptidase. From the k_{cat} values, we can also reach the conclusion that the enzyme preferred to hydrolyze Leu-MCA rather than Arg-MCA. Thus, the enzyme was regarded as a LAP. The substrate specificity of the present enzyme was quite similar to that of common carp LAP; however, the K_m value of the present LAP was 1.55 μM, which is lower than that of common carp LAP (4.6 μM) for Leu-MCA (*21*). It has been reported that the substrate specificity for amino acid−MCA of an aminopeptidase may not be in accordance with that for oligopeptides (*9*). This suggests that aminopeptidase cleavage of the peptide-bound substrates in vivo may not be completely consistent with that of the amino acid−MCA substrates. For example, human placental leucine aminopeptidase could cleave natural bioactive neuropeptides such as met-enkephalin, dynorphin A, neurokinin A, neuromedin B, and somatostatin (*30*). In general, LAPs prefer to hydrolyze nonpolar aliphatic (Leu, Ile, Ala, and Val), basic (Arg), and sulfur-containing (Met) amino acid residues (*15*), which may improve the taste of meat. Although the enzyme does not degrade myofibrillar proteins, its contribution to free amino acid increase during post-mortem aging is noteworthy (*12*). This contribution is especially important in the production of the Chinese traditional condiment fish sauce, where extensive fermentation in the presence of microorganisms and various endogenous proteinases (including aminopeptidases) is necessary to enhance fish muscle proteins to degrade into small peptides and free amino acids.

The LAP was mostly inhibited by metalloproteinase inhibitor EDTA and 1,10-phenanthroline and moderately inhibited by EGTA, whereas the serine proteinase inhibitor PMSF only slightly suppressed the activity. E-64, a specific inhibitor for cysteine proteinase, showed considerable inhibition. The reason for this phenomenon was proposed to be the presence of a critical cysteine group(s) near the active site of the enzyme. Bestatin, a general inhibitor of aminopeptidase, was also a simple intersecting linear competitive inhibitor of the LAP, and its K_i was 1.44 μM, which was in agreement with that of *A. pisum* aminopeptidase (K_i = 1.8 μM) (*23*).

Many, but not all, aminopeptidases are metalloenzymes that contain a central Zn^{2+}, which is essential for enzyme activity (*14*). LAP in bovine lens uses Mn^{2+} and Zn^{2+} as its metal cofactors. LAP from halophilic bacterium *Bacillus* sp. N2 was activated only by Co^{2+} (*27*), whereas LAP from human liver was activated by a series of divalent metal ions including Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, and Fe^{2+} (*6*). Only Mg^{2+} restored the activity of rat brain neutral aminopeptidase (*3*). All of these facts suggested that LAPs constitute a diverse set of exopeptidases with variable divalent cation requirements. The LAP purified in the present study was sensitive to the proteinase inhibitor EDTA and 1,10-phenanthroline; furthermore, Zn^{2+} and Mn^{2+} could almost fully restore the apoenzyme's activity dialyzed by EDTA. These results strongly suggested that the enzyme uses Zn^{2+} and (or) Mn^{2+} as its metal cofactor(s). However, the activity of the enzyme was inhibited by 1.0 mM

Zn^{2+} or Mn^{2+} (**Table 3B**), suggesting that an excess amount of Zn^{2+} or Mn^{2+} may change the protein conformation of its metal-binding site and inhibit its activity, just as 47b LAP is inhibited by the excess amount of Co^{2+} (*17*).

It has been reported that aminopeptidase does not possess a signal peptide necessary for passage into the secretory pathway, and therefore the enzyme most likely functions intracellularly (*31*). Western blot results showed that LAP is widely distributed in red sea bream tissues including skeletal muscle, brain, liver, kidney, gut, stomach, pyloric ceca, and gill (**Figure 7B**). The granular pattern of the immunoreactivity revealed that the LAP is localized in vesicles of the skeletal muscle cell (**Figure 8B**). A similar result was also found in placental leucine aminopeptidase (*30*). Although the specific role of LAPs is not well-known, their expression in different tissues is consistent with their role in a variety of processes such as the maturation of proteins, the terminal degradation of proteins, and the metabolism of secreted regulatory molecules (*31*).

In conclusion, our present paper describes the purification and characterization of a LAP for the first time from marine fish red sea bream skeletal muscle. LAP is widely distributed in various tissues and exhibited an intracellular location in skeletal muscle cell. The role that the LAP plays in myofibrillar protein metabolism in vivo and its exact function, however, remain unclear. The potential application and function of aminopeptidases such as LAP in the production of the Chinese traditional condiment fish sauce are noteworthy.

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