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Purification and characterization of a leucine aminopeptidase from the skeletal muscle of common carp (*Cyprinus carpio*)

Bing-Xin Liu [a], Xue-Li Du [a], Li-Gen Zhou [b], Kenji Hara [c], Wen-Jin Su [a], Min-Jie Cao [a,*]

[a] *College of Biological Engineering, The Key Laboratory of Science and Technology for Aquaculture and Food Safety, Jimei University, Jimei, Xiamen 361021, China*
[b] *Institute of Food Processing, Zhejiang Academy of Agriculture Sciences, Hangzhou 310021, China*
[c] *Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan*

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Abstract

An aminopeptidase was purified from the skeletal muscle of common carp (*Cyprinus carpio*) to homogeneity, with 1160-fold purification and a yield of 4.3%. The purification procedure consisted of ammonium sulfate fractionation and sequential chromatographic steps, including DEAE-Sephacel, Sephacryl S-200, hydroxyapatite, Phenyl Sepharose and Macro-Prep High Q columns. The molecular mass of the enzyme was estimated to be 105 kDa and 100 kDa by SDS-PAGE under reducing conditions and gel filtration chromatography, respectively, suggesting it to be a monomer. The enzymatic activity was optimal at 35 °C and pH 7.0. The metal-chelating agents EDTA, EGTA and 1,10-phenanthroline specifically inhibited the enzyme activity while inhibitors of other proteinases did not show much effect, indicating that it was a metalloproteinase. Furthermore, bestatin, a specific aminopeptidase inhibitor strongly inhibited its activity. Divalent cations Mn^{2+}, Mg^{2+} and Ba^{2+} slightly enhanced the enzymatic activity, while Co^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity to different extents. In addition, a sulfhydryl reagent was necessary to maintain its activity. Substrate specificity study revealed that the purified enzyme preferentially hydrolyzed Leu-MCA, followed by Arg-MCA, Ala-MCA and Tyr-MCA and it was thus regarded as a leucine aminopeptidase (LAP, EC 3.4.11.1). The apparent K_m and V_{max} values of the enzyme were 4.6 μM and 9.6 μmol min^{-1} mg^{-1}, respectively for substrate Leu-MCA. This is the first report concerning purification and characterization of LAP from freshwater fish.

Keywords: Common carp; Purification; Leucine aminopeptidase

1. Introduction

Aminopeptidases catalyze the cleavage of amino acids from the amino terminus of proteins or peptides. They are widely distributed throughout the animal, plant and microorganism kingdoms (Taylor, 1993). Aminopeptidases are vital for metabolic pathway regulation, cell maturation and turnover of proteins, including utilization of exogenous proteins as nutrient substances and elimination of non-functional proteins (Gonzales & Robert-Baudouy, 1996).

In muscle tissues, different types of aminopeptidases have been identified, including leucine aminopeptidase (LAP) from human (Mantle, Lauffart, & Gibson, 1991), aminopeptidase B and C from porcine muscle (Flores, Aristoy, & Toldrá, 1993; Nishimura, Kato, Rhyu, Okitani, & Kato, 1992) and aminopeptidase H from chicken (Rhyu, Nishimura, Kato, Okitani, & Kato, 1992). More recently, aminopeptidase D was purified from bovine skeletal muscle (Migita & Nishimura, 2006). Aminopeptidases, together with other endogenous proteinases, are believed to play

* Corresponding author. Tel.: +86 592 6180378; fax: +86 592 6180470.
E-mail address: mjcao@jmu.edu.cn (M.-J. Cao).

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important roles in muscle autolysis during *post mortem* storage. Initial degradation of myofibrillar proteins into oligopeptides was performed by endopeptidases, including calpains (Bartoli & Richard, 2005) and cathepsins B and L (Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003). This results in the breakdown of muscle structure, causing muscle softening (Ladrat et al., 2003). Further degradation of oligopeptides into free amino acids was carried out by exopeptidases, such as aminopeptidases and carboxypeptidases (Migita & Nishimura, 2006; Toldrá & Flores, 1998).

Leucine aminopeptidases (LAPs), a class of cellular exoproteases, preferably catalyze the hydrolysis of a leucine residue from the amino-termini of proteins or peptides. LAPs are often viewed as cell maintenance enzymes with critical roles in turnover of peptides (Matsui, Fowler, & Walling, 2006). 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 (Matsui et al., 2006). LAPs in animals are ubiquitous and have been identified in tissues, including muscle (Mantle et al., 1991), liver (Kohno, Kanda, & Kanno, 1986) and lens (Kim & Lipscomb, 1993). The roles of LAP in plants concerning defence, membrane transport of auxin receptors and meiosis, have also been reported (Gu & Walling, 2002; Matsui et al., 2006). In microbes, LAPs serve as transcriptional repressors to control pyrimidine, alginate and cholera toxin biosynthesis, as well as mediating site-specific recombination events in plasmids and phages (Matsui et al., 2006). Though different column chromatographies have been applied to purification of native LAPs, immunoaffinity column chromatography seems the most efficient way (Nakanishi et al., 2000). More recently, the production of LAP from microorganisms by fermentation has been paid considerable attention due to its debittering function in the food industry (Chien et al., 2002; Deejing, Yoshimune, Lumyong, & Moriguchi, 2005).

In contrast, investigations on aminopeptidases from fish are very rare. Till now, only aminopeptidases from Alaska pollack roe and tuna pyloric caeca have been purified (Chiou, Matsui, & Konosu, 1989; Hajjou & Le Gal, 1994), while aminopeptidase from freshwater fish has never been reported. In this study, we describe the purification and characterization of an LAP (cmLAP) from the skeletal muscle of common carp.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Cultured common carp (*Cyprinus carpio*) (body weight 600–800 g) were purchased from the fish market of Jimei, Xiamen, and transported to our laboratory alive. The fish were killed by decapitation, eviscerated and washed; skeletal muscle was collected.

2.1.2. Chemicals

DEAE-Sephacel, Sephacryl S-200 HR and Phenyl Sepharose 6 Fast Flow were purchased from Amersham Biosciences (Uppsala, Sweden). Econo-Pac Macro-Prep High Q column, hydroxyapatite, molecular weight calibration marker for gel filtration and dithiothreitol (DTT) were from Bio-Rad (Hercules, CA, USA). L-Arginine-4-methyl-coumaryl-7-amide hydrochloride (Arg-MCA), *t*-butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and *t*-butyloxy-carbonyl-Leu-Arg-Arg-4-methyl-coumaryl-7-amide (Boc-Leu-Arg-Arg-MCA) were obtained from the Peptide Institute (Osaka, Japan). Other fluorogenic substrates (MCA substrates), bestatin hydrochloride, bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF) and 1,10-phenanthroline monohydrate, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were products of Sigma (St Louis, MO, USA). L-3-Carboxy-*trans*-2,3-epoxy-propionyl-L-leucin-4-guanidinobutylamide (E-64) was obtained from Amresco (Solon, OH, USA). Pepstatin was from Roche (Mannheim, Germany). Protein marker for SDS-PAGE was from Fermentas (Lithuania). Other reagents were of analytical grade.

2.2. Assay of aminopeptidase activity

Routinely, aminopeptidase activity was measured using Leu-MCA as substrate according to the method of Umetsu et al. (2003) with some modifications. Appropriately diluted enzyme (100 μl) was added to 850 μl of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.02% NaN_3 (buffer A). Fifty microliter of 10 μM substrate were added to initiate the reaction. The reaction was performed at 37 °C for 10 min and stopped by addition of 1.5 ml of stopping agent (methyl alcohol:*n*-butyl alcohol:distilled water = 35:30:35, 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 emission wavelength of 450 nm on a fluorescence spectrophotometer (FP-6200, Jasco, Japan). One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 nmol of AMC per min. Routinely, proteolytic activity assays were performed in duplicate and variation between duplicate samples was always <5%. The mean values were used.

2.3. Purification of aminopeptidase

All procedures were performed at 4 °C. About 500 g of minced fish muscle were homogenized with 3-fold weight of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT), 0.02% NaN_3 and 1 mM PMSF, using a homogenizer (Kinematica, PT-2100, Switzerland), and centrifuged at 10,000*g* for 15 min in a centrifuge (Avanti J-25, Beckman Coulter, USA). After centrifugation (10,000*g*, 15 min), the supernatant was fractionated with ammonium sulfate from 40% to 60%

saturation and the resulting precipitate was collected and dissolved in a minimum volume of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.02% NaN_3 (buffer A), and extensively dialyzed against the same buffer. The dialysate was subsequently applied to a DEAE-Sephacel column (2.5 × 25 cm) previously equilibrated with buffer A. Contaminating proteins were removed by washing the column with starting buffer until the absorbance at 280 nm reached baseline. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer A in a total volume of 600 ml at a flow rate of 1.0 ml/min. Active fractions eluted by NaCl gradient at a concentration of approximately 0.25 M were pooled and concentrated by ultrafiltration with a membrane of YM-10 (Millipore, MA, USA) and 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 hydroxyapatite column (2.5 × 5 cm) which was previously equilibrated with buffer A. The enzyme was eluted with a linear gradient of sodium phosphate buffer (pH 7.0) containing 1 mM DTT and 0.02% NaN_3 from 25 to 100 mM in a total volume of 80 ml at flow rate 0.4 ml/min. Active fractions from hydroxyapatite column were further dialyzed against buffer A containing 1 M ammonium sulfate and applied to a Phenyl Sepharose 6 Fast Flow column (0.8 × 4 cm) equilibrated with the dialysis buffer. The proteins retained were eluted by a linear gradient of ammonium sulfate from 1 M to 0 M in buffer A in a total volume of 50 ml at a flow rate of 0.4 ml/min. Active fractions were further collected, dialyzed against buffer A containing 50 mM NaCl, and loaded onto the Econo-Pac Macro-Prep High Q column (1 ml) equilibrated with buffer A. After elution with a linear gradient of NaCl from 0.05 to 0.5 M, in a total volume of 30 ml, active fractions were collected and used for SDS-PAGE analysis and enzymatic characterization.

2.4. Protein concentration determination

The absorbance at 280 nm was used to monitor the protein after column chromatographies. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard.

2.5. 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 on a 10% polyacrylamide gel according to the method of Laemmli (1970); the gel was stained with Coomassie Brilliant Blue R-250.

Gel filtration 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 (670,000 Da), bovine γ-globulin (158,000 Da), chicken ovalbumin (44,000 Da), equine myoglobin (17,000) and vitamin B_{12} (1350 Da) as standards.

2.6. Effects of pH and temperature

The effect of pH on the LAP was determined at a temperature of 37 °C using Leu-MCA as substrate in 50 mM 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 9.0–11.0). Optimal temperature was measured in buffer A at different temperatures (15–60 °C).

2.7. Proteolytic effect of the enzyme on bovine serum albumin

To investigate the hydrolyzing activity of the enzyme toward protein substrate, purified enzyme (0.5 units) was mixed with bovine serum albumin at a final concentration of 750 μg/ml in buffer A and incubated at 37 °C for different time intervals. The degradation result was determined by SDS-PAGE.

2.8. Effects of protease inhibitors

To investigate the effects of different proteinase inhibitors on the enzyme, purified enzyme was preincubated with buffer A, together with corresponding inhibitors at different final concentrations, for 30 min at room temperature. Remaining activity was determined by the method described above. Control tests were performed in the absence of inhibitor.

2.9. Effects of metal ions or sulfhydryl reagent

To investigate the effect of metal ions on the enzyme, purified enzyme was preincubated with different cations at appropriate final concentrations for 30 min at room temperature and the remaining activity was determined.

To clarify whether the purified enzyme requires sulfhydryl reagent or not, DTT was removed from the enzyme by extensive dialysis against 25 mM sodium phosphate buffer (pH 7.0) containing 0.02% NaN_3 for 24 h. DTT was subsequently added to the enzyme solution to different final concentrations and the remaining activity was measured after incubating the solution for 30 min at room temperature. Control tests were performed in the absence of DTT.

2.10. Determination of kinetic parameters

Kinetic parameters of the purified enzyme were estimated for substrate Leu-MCA at concentrations ranging from 1 to 20 μM. Kinetic parameters were calculated from the Lineweaver-Burk plots.

3. Results and discussion

3.1. Enzyme purification

In the present study, a leucine aminopeptidase (cmLAP) was identified in the sarcoplasmic fraction of common carp skeletal muscle and purified to homogeneity through six purification procedures. The aminopeptidase activity revealed a single peak when eluted from DEAE-Sephacel column by NaCl gradient, which corresponds to the second protein peak (Fig. 1A). This purification step removed most contaminating proteins and yielded an obvious increase in specific activity. Active fractions were concentrated and separated by gel filtration chromatography in a Sephacryl S-200 column which allowed the removal of proteins with large molecular masses (data not shown). The following hydroxyapatite column was a critical step for removing contaminating proteins (Fig. 1B). The next hydrophobic interaction column, Phenyl Sepharose, removed a contaminating protein with Mr of 115 kDa. After the final column chromatography on Econo-Pac Macro-Prep High Q, the enzyme was finally purified to homogeneity (Fig. 1C). A summary of the purification is shown in Table 1. The whole purification procedure resulted in a 1160-fold increase in specific activity with a 4.3% recovery. To identify the purification efficacy, fractions obtained from each purification step were analyzed by SDS-PAGE. As shown in Fig. 2A, the final enzyme preparation revealed as a single band with Mr of approximately 105 kDa.

3.2. Characterization of cmLAP

3.2.1. Molecular mass of cmLAP

A gel filtration chromatography analysis revealed the purified cmLAP with a molecular weight of approximately 100 kDa (Fig. 2B), which is in accordance with that estimated by SDS-PAGE (105 kDa), suggesting that cmLAP is a monomer. This molecular mass value is similar to aminopeptidases from rat brain (102 kDa) (Wagner, Tavianini, Herrmann, & Dixon, 1981) and Alaska pollack roe (105–125 kDa) (Chiou et al., 1989). It is smaller than an aminopeptidase from tuna pyloric caeca (150 kDa) (Hajjou & Le Gal, 1994) and most LAPs from mammals and plants as these enzymes are hexamers composed of ~60 kDa subunits (Gu & Walling, 2002; Kohno et al., 1986; Matsui et al., 2006). However, it is higher than those from *Bacillus* sp. N2 (58 kDa) (Lee, Chun, Kho, & Chun, 1998) and *Aspergillus sojae* (37 kDa) (Chien et al., 2002).

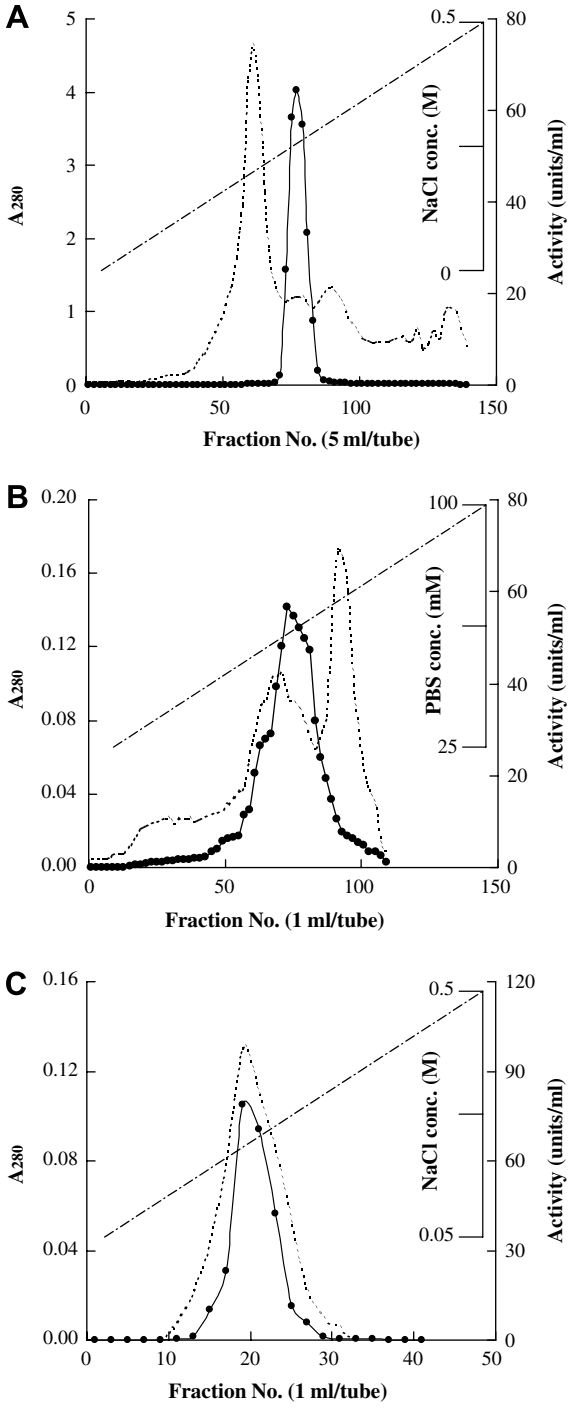

Fig. 1. Column chromatography purification of leucine aminopeptidase from carp. (A) DEAE-Sephacel chromatography; (B) hydroxylapatite column chromatography; (C) Econo-Pac Macro-Prep High Q column. Absorbance at 280 nm (---); Leu-MCA -hydrolyzing activity (●).

3.2.2. Effects of pH and temperature

As shown in Fig. 3A, cmLAP was most active at pH 7.0 suggesting that it is a neutral aminopeptidase. The activity was negligible below pH 5.5 and above pH 9.0, suggesting that the enzyme did not function efficiently in acidic and alkaline environments. The enzyme revealed an optimum temperature of 35 °C and the activity obviously decreased above 40 °C (Fig. 3B). For routine enzymatic activity assay, a temperature of 37 °C was used, which was higher than the temperature used by Umetsu et al. (2003). The optimal temperature difference was quite possibly caused by enzyme characteristics as the aminopeptidase purified by Umetsu et al. (2003) hydrolyzed Ala-MCA most efficiently while the present enzyme cleaved Leu-MCA with

Table 1
Summary of purification of the aminopeptidase from carp ordinary muscle

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	6930	8706	1.3	100	1.0
$(NH_4)_2SO_4$ precipitation	1405	2616	1.9	30.1	1.5
DEAE-Sephacel	13.2	1727	131	19.9	101
Sephacryl S-200	2.4	904.8	377	10.4	290
Hydroxylapatite	0.5	694.8	1311	8.0	1008
Phenyl Sepharose	0.3	426.1	1420	4.9	1093
High-Q	0.25	377.1	1,508	4.3	1160

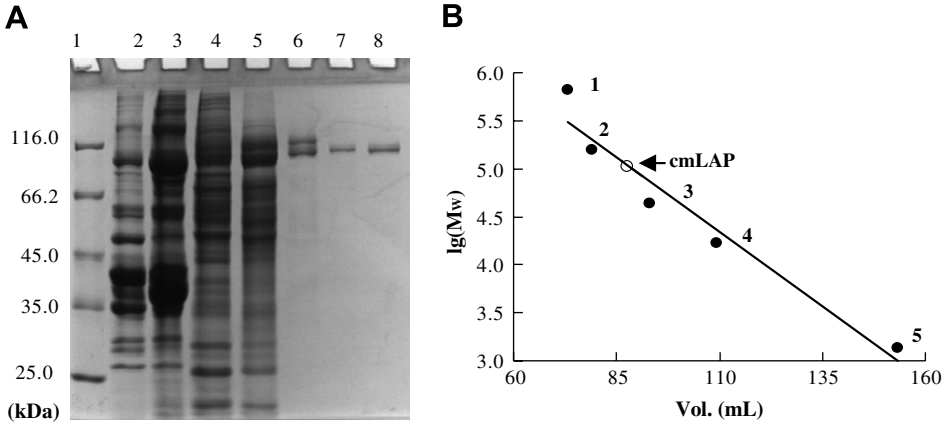

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified cmLAP (A) and its native molecular mass on Sephacryl S-200 gel filtration. (A) Purified cmLAP was subjected to electrophoresis on a 10% gel, followed by Coomassie brilliant blue staining. Lane 1, molecular weight marker; lane 2, crude extract; lane 3, 40–60% ammonium sulfate precipitation; lane 4, DEAE-Sephacel fraction; lane 5, Sephacryl S-200 fraction; lane 6, hydroxylapatite fraction; lane 7, Phenyl Sepharose fraction; lane 8, Macro-Prep High Q fraction. (B) Sephacryl S-200 gel filtration estimation of molecular mass: 1, thyroglobulin; 2, bovine γ-globulin; 3, chicken ovalbumin; 4, equine myoglobin; 5, vitamin B_{12}. Arrow head indicates the elution position (open circle) of cmLAP.

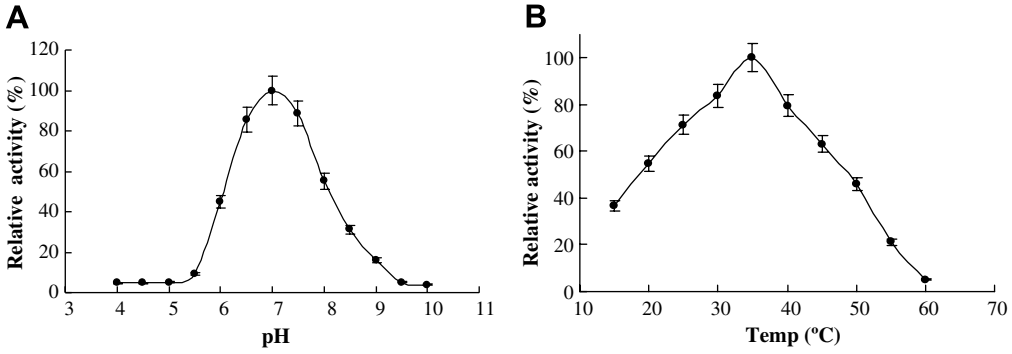

Fig. 3. Optimal temperature (A) and pH (B) of cmLAP. The enzyme activity was determined using Leu-MCA as substrate.

the highest rate. It is noteworthy that the enzyme retained 36.9% of its optimal activity even at 15 °C. This temperature-dependent characteristic may be ascribed to the accommodation temperature of common carp. Accordingly, it can be presumed that the cmLAP may play an active role, not only *in vivo* toward physiological substrates, but also in collaboration with other proteinases, such as cathepsins that functions in further degradation of peptides during the period of *post mortem* cold storage.

Unlike mammalian animal meats in which *post mortem* aging is necessary for flavour enhancement (Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004), *post mortem* tenderization in fish muscle is one of the most unfavourable quality changes. Therefore, extensive biochemical characterizations of proteinases involved in this process are essential in order 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 calpains and cathepsins B and L. Calpains are optimally active at neutral pH and have a broad pH-dependency range, while cathepsins B and L are optimally active at pH 5.0–6.0.

Calpains activated by released Ca^{2+} hydrolyze muscle structural proteins. Calpain 1, having a tight association with myofibrils, degrades desmin, nebulin, troponin T and a giant cytoskeletal protein, titin, while calpain 3 associates with titin and these two enzymes are responsible for the initial steps of myofibrillar disassembly, starting with the destruction of Z lines at neutral pH (Raynaud et al., 2005). Cathepsins localized in the lysosome, with acidic environment, are involved in the further proteolysis of *post mortem* muscle where the pH is decreased to 5.5–5.7 (Ladrat et al., 2003). Though the physiological role of cmLAP in fish muscle still remains unclear, during *post mortem* tenderization of fish muscle, like other aminopeptidases (Rhyu et al., 1992), cmLAP is also responsible for the hydrolysis of peptides produced by the processing of cathepsins to free amino acids when the pH of muscle returns to neutral by the release of amine products.

3.2.3. Substrate specificity

In order to clarify the substrate specificity of the purified enzyme, its activities toward various MCA substrates were measured and the results are presented in Table 2. Among the substrates tested, the enzyme preferred Leu-MCA most. It also hydrolyzed Arg-MCA and Ala-MCA significantly. In addition, Tyr-MCA and Val-MCA were also hydrolyzed by cmLAP, though with relatively low hydrolysis rates of 35.7% and 6.3%, respectively. On the basis of substrate specificity, the enzyme was regarded as a leucine aminopeptidase (LAP). It has been reported that LAPs often reveal a broader preference for cleaving hydrophobic amino acid residues and this substrate selectivity might indicate their specific roles in the metabolism of normal or pathological muscle *in vivo* (Matsui et al., 2006). With characteristics of an aminopeptidase, purified cmLAP did not reveal any endoproteinase activity toward N-terminus-blocked substrates, i.e. Boc-Phe-Ser-Arg-MCA and Boc-Leu-Arg-Arg-MCA. cmLAP also did not exhibit apparent proteolytic activity toward protein substrates such as bovine serum albumin (Fig. 4) and myofibrils (data not shown), further confirming that it is an exopeptidase. Though cmLAP does not degrade myofibrillar proteins as effectively as endopeptidases, especially cathepsins, its contribution to free amino acid increase during *post mortem* aging is noteworthy. This contribution is especially important in the production of 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 degradation into small peptides and free amino acids.

Table 2
Substrate specificity of purified aminopeptidase activity

Substrate	Relative activity (%)
Leu-MCA	100
Arg-MCA	62.5
Ala-MCA	47.8
Tyr-MCA	35.7
Pro-MCA	8.0
Val-MCA	6.3
Gly-MCA	1.8
Boc-Phe-Ser-Arg-MCA	0
Boc-Leu-Arg-Arg-MCA	0

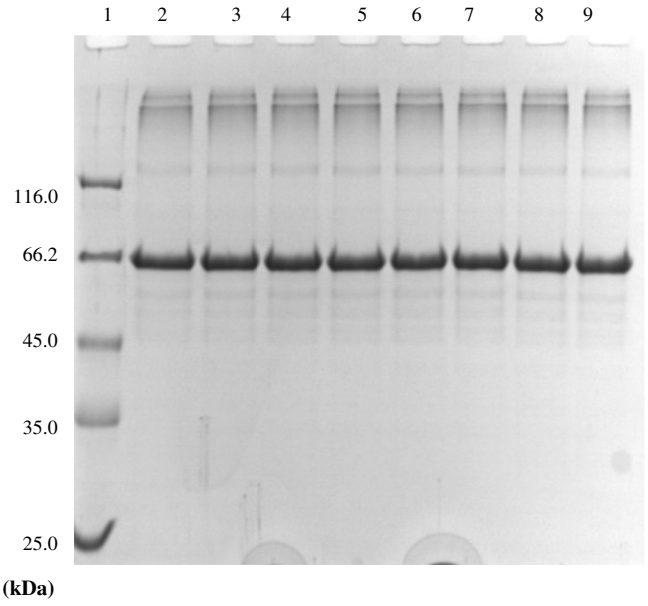

Fig. 4. Proteolytic effect of the enzyme on bovine serum albumin. Lane 1, molecular weight marker; lane 2, control; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 1 h; lane 7, 1.5 h; lane 8, 2 h; lane 9, 3 h.

3.2.4. Effect of protease inhibitors

The effect of potential inhibitors on cmLAP is shown in Table 3. Like most known aminopepetidases, cmLAP seems to have a metal ion in its active site since metal-chelating reagents, such as EDTA, EGTA and 1,10-phenanthroline, strongly inhibited the activity. Bestatin, a

Table 3
Effects of chemicals and protease inhibitors on the enzyme activity

Inhibitor	Concentration (mM)	Residual activity(%)
EDTA	1	25.3
	5	23.2
	10	21
EGTA	1	16.1
	5	9.1
	10	4.0
o-Phenanthroline	0.2	4.0
	1	3.5
	5	2.4
Bestatin	0.01	5.9
	0.1	0
PMSF	1	95.4
	5	87.4
Pepstatin	0.03	87.2
	0.15	81.5
E-64	0.3	70

competitive and specific inhibitor of leucine aminopeptidase, aminopeptidase B and triamino peptidases, was quite effective on cmLAP at concentrations as low as 5 μM (Fig. 5), and it completely restrained the cmLAP activity at 0.1 mM. In contrast, inhibitors of serine proteinase (PMSF), asparatic proteinase (pepstatin) and cysteine proteinase (E-64) did not show much inhibitory effect. This result is consistent with the characteristic that LAPs are metallopeptidases (Matsui et al., 2006).

3.2.5. Effects of metal ions and sulfhydryl reagent

As shown in Table 4, the enzyme was strongly inactivated by metal ions of Co^{2+}, Cu^{2+} and Zn^{2+} and partially by Ca^{2+} and Fe^{2+}. In contrast, it was slightly activated by Mn^{2+} and Mg^{2+} at concentrations from 0.1 mM to 1 mM. In addition, Ba^{2+} at 0.1 mM caused slight stimulation of the enzyme activity while, at 1 mM, partial inactivation was observed. Similar effects of Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} on LAP from swine liver have also been reported (Ledeme, Hennon, Vincent-Fiquet, & Plaquet, 1981).

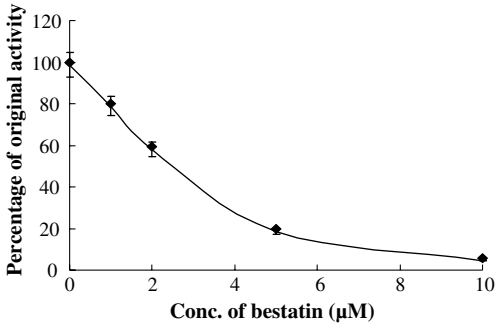

Fig. 5. Inhibitory effect of bestatin on cmLAP. cmLAP was preincubated with different concentrations of bestatin at room temperature for 30 min, and the remaining activity was determined.

Table 4
Effect of metal ions on the enzyme activity

Metal ions	Concentration (mM)	Residual activity (%)
None	0	100
$MnCl_2$	0.1	109.2
	1	104
$MgCl_2$	0.1	98.4
	1	109.2
$BaCl_2$	0.1	107.9
	1	85
$FeSO_4$	0.1	99.4
	1	75.2
$CaCl_2$	0.1	95
	1	72
$ZnSO_4$	0.1	62.3
	1	27.3
$CuSO_4$	0.1	91.7
	1	14.8
$CoCl_2$	0.1	52.1
	1	9.6

Although many LAPs bind mainly zinc ion (Taylor, 1993), and two metal binding sites, the tightly binding and readily exchanging metal binding site in bovine lens LAP and tomato LAP have been identified by X-ray crystallography (Gu & Walling, 2002; Kim & Lipscomb, 1993), there are some exceptions. LAPs from Bacillus stearothermophilus and the halophilic bacterium *Bacillus* sp. N2, were activated only by Co^{2+} (Kuo, Hwang, Lai, Yang, & Lin, 2003; Lee et al., 1998), while 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+} (Kohno et al., 1986). LAP in bovine lens uses Mn^{2+} and Zn^{2+} as its metal cofactors (Cappiello et al., 2004). All these facts suggested that LAPs constitute a diverse set of exopeptidases with variable divalent cation requirements. cmLAP purified in the present study seems to use Mn^{2+} and(or) Mg^{2+} as its metal cofactor(s).

Addition of 1 mM DTT was found to be necessary during isolation of the aminopeptidase. The enzymatic activity decreased completely once the purified enzyme was dialyzed to remove DTT. This is similar to the aminopeptidase from Alaska pollack roe, suggesting that free sulfhydryl groups are vital for the conformational maintenance of the enzyme (Chiou et al., 1989). Although the molecular mass, optimal temperature and pH of cmLAP are similar to those of the aminopeptidase from Alaska pollack roe (Chiou et al., 1989), their substrate specificity is different; cmLAP prefers the L-leucine residue at the N-terminal, while the latter hydrolyzed L-alanine most. Furthermore, their sensitivities to the proteinase inhibitor EDTA were also different. Only 25.3% of cmLAP activity remained in the presence of 1 mM EDTA while the aminopeptidase from Alaska pollack roe retained 93% activity under the same conditions. In addition, the aminopeptidase from tuna pyloric caeca is obviously different from cmLAP as it is a dimer with two subunits of 72 kDa and reveals optimal activity at pH 8.8 and 65 °C (Hajjou & Le Gal, 1994). Thus, the present results indicate that cmLAP is a novel leucine aminopeptidase from fish muscle.

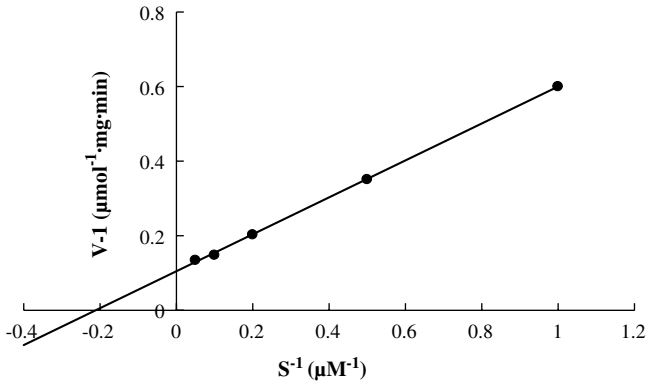

Fig. 6. Lineweaver-Burk doule reciprocal plot of cmLAP.

3.2.6. Kinetic parameters

The Lineweaver-Burk plot of cmLAP using Leu-MCA as substrate is shown in Fig. 6. The K_m and V_{max} were 4.6 μM and 9.6 μmol mg^{-1} min^{-1}, respectively.

4. Conclusion

A novel cmLAP with molecular mass of approximately 100 kDa was identified and purified to homogeneity from the skeletal muscle of common carp. The divalent cation Mn^{2+} is the most potent cofactor of the enzyme. The role that cmLAP plays in myofibrillar protein metabolism *in vivo* remains unclear. Though cmLAP degrades various exopeptidase MCA substrates, it does not decompose bovine serum albumin and myofibrils, suggesting that its contributions to peptide lysis in *post mortem* fish muscle are less than those of cathepsins, especially cathepsins B and L. However, the application and function of aminopeptidases such as cmLAP in the production of Chinese traditional condiment fish sauce is noteworthy. In conclusion, our present manuscript for the first time identifies the existence of leucine aminopeptidase (LAP) in the skeletal muscle of freshwater fish. This may aid further study of such enzymes, and especially their potential application in fish sauce production.

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