Process Biochemistry 50 (2015) 1061–1067

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Purification and biochemical characterization of a puromycin-sensitive aminopeptidase from black carp muscle

Si-Wei Li[a,1], Li-Ping Lin[a,1], Su-Hua Chen[b], Meng-Yu Fu[a], Guo-Ping Wu[a,*]

[a] College of Food Science and Engineering, Jiangxi Agricultural University, Nanchang 330045, China
[b] College of Environmental and Chemical Engineering, Nanchang Hangkong University, Nanchang 330063, China

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 20 March 2015
Accepted 7 April 2015
Available online 15 April 2015

Keywords:
Black carp
Salted fish
Aminopeptidase
Purification
Biochemical characterization

ABSTRACT

Exopeptidases such as aminopeptidases and carboxypeptidases are believed to contribute to the formation of free amino acids during postmortem storage and the processing of meat. In order to understand the role of aminopeptidases in the generation of amino acids and formation of flavor compounds in Chinese traditional salted fish, an aminopeptidase was purified and characterized from black carp muscle. The peptide mass fingerprinting of this enzyme suggested that it was a puromycin-sensitive aminopeptidase purified to homogeneity by ammonium sulfate fractionation and three chromatographies. Puromycin was further confirmed as a competitive inhibitor with K_i value of 0.25 μM. The 100-kDa enzyme preferentially hydrolyzed substrate Lys-MCA with optimum temperature and pH at 40 °C and 7.5, respectively. At the concentration of 3.2% NaCl and 5% ethanol, the enzyme remained 58.5% and 87.5% of its initial activity, respectively. The aminopeptidase(s) activity decreased slowly and remained 41.1% of its initial activity even after 12 days salting of black carp muscle. These results suggest the possible contribution of fish aminopeptidases to free amino acid formation and flavor generation in Chinese traditional salted fish.

1. Introduction

Salting of food, particularly the salting of fish, is an ancient food preservation technique still in use around the world [1]. Layú, the traditional name for salted fish produced in southern China, is popular in the southern and eastern parts of China because of its special chewiness, flavor satisfying characteristics, and much longer shelf life [2]. Black carp (*Mylopharyngodon piceus*) and grass carp (*Ctenopharyngodon idellus*) are two species of freshwater fish that are usually used for Layú in China, due to their huge stocks, high quality proteins (15–26%, w/w) and relatively low market price. Generally, cleaned fish will be mixed with salt in a 10:1 or 20:1 ratio (fish:salt, w/w), depending on the desired final product taste. Then, salt-mixed fish are subjected to fermentation. After 15–20 days of maturing, it is suitable for consumption and can be stored for 2–3 months [2].

Five major factors are believed to influence the quality of the final product of salted fish: fish species, type of salt, the ratio of fish and salt, minor ingredients, and salting method [3]. The type of fish species also affects the type of proteins that serve as nutrients for microorganisms and substrates for enzymes, both of which hydrolyze proteins into small peptides and amino acids. Proteins are highly complex polymers made of up to 20 amino acids [4]. Muscle proteins in salted fish can be hydrolyzed into small peptides and amino acids, which contribute to the specific aroma and flavor in the fermentation of fish [1].

Proteolysis and lipolysis constitute two of the most important biochemical mechanisms occurring during the processing of salted fish with respect to the final sensory quality. Proteolysis contributes to the texture by breakdown of the muscle structure, to the taste through the generation of small peptides and free amino acids and to the aroma by further degradation of some free amino acids [5–7]. Although the amino acid composition of fresh fish does not vary greatly; however, the free amino acid composition changes considerably after processing and storage. There are several consecutive stages in proteolysis: (a) breakdown of major myofibrillar proteins by the action of endopeptidases, such as calpains and cathepsins, (b) generation of polypeptides that act as substrates for peptidases to generate small peptides and (c) intense generation of free amino acids by the action of exopeptidases like aminopeptidases and carboxypeptidases [5,8]. The generation of free amino acids is important because they directly contribute to taste. The contributions of cathepsins and calpains have been widely studied, but

* Corresponding author. Tel.: +86 791 83828420; fax: +86 791 83828420.
E-mail address: jdwgp@163.com (G.-P. Wu).
[1] These authors contributed equally to this manuscript.

http://dx.doi.org/10.1016/j.procbio.2015.04.002
1359-5113/

there exists a limited knowledge about the role of aminopeptidases in the generation of free amino acids and their contribution to flavor especially in Chinese traditional salted fish such as Layú [4,9].

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 are 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 [9,10]. These enzymes are considered to have their original substrate specificities dependent on their physiological roles and are widely distributed throughout animals, plants, and microorganisms. They are vital for metabolic pathway regulation, cell maturation and turnover of proteins [11].

Fish aminopeptidases from Alaska Pollack roe and tuna pyloric caeca have been reported on [12,13]. More recently, we described the purification of a leucine aminopeptidase from the skeletal muscle of the common carp [14], red sea bream [15] and a lysine aminopeptidases from Japanese flounder [16]. The objective of this study was to purify and characterize an aminopeptidase from the skeletal muscle of black carp, and trace the changes of aminopeptidase activity in fish muscle during salting and storage. The possible contributions of aminopeptidases to the generation of free amino acids and flavor development are discussed regarding Chinese traditional salted fish.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Cultured black carp (*M. piceus*) (body weight 1000–2000 g) were purchased alive from a local fisheries store and were cooled in ice-water before killing. They were then killed by decapitation, eviscerated and washed, and the skeletal muscle was collected.

2.1.2. Chemicals

DEAE-Sephacel and Phenyl-Sepharose 6-Fast Flow were purchased from Amersham Biosciences (Uppsala, Sweden). Econo-Pac CHT-II Cartridge hydroxyapatite column and dithiothreitol (DTT) were from Bio-Rad (Hercules, CA). L-Arginine-4-methylcoumaryl-7-amidehydrochloride (Arg-MCA), L-Lysine-4-methylcoumaryl-7-amide (Lys-MCA), t-Butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and t-Butyloxy-carbonyl-Val-Leu-Lys-4-methyl-coumaryl-7-amide (Boc-Val-Leu-Lys-MCA) were obtained from the Peptide Institute (Osaka, Japan). Other fluorogenic substrates (Leu-MCA, Ala-MCA, Tyr-MCA, Pro-MCA, Val-MCA and Gly-MCA), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin and were purchased from Sigma (St. Louis, MO, USA). A protein marker for SDS-PAGE was bought from Fermentas (Vilnius, Lithuania). Other reagents were all of analytical grade.

2.2. Assay of aminopeptidase activity

Aminopeptidase activity was measured using Arg-MCA as substrate according to the method of Zhang et al. [17] with some modification unless otherwise stated. In brief, diluted enzyme (10 μl) was added to 940 μl of 25 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM DTT (buffer A). Thereafter, 50 μl of substrate (10 μM) was added and the reaction was performed at 37 °C for 10 min. Reactions were stopped by addition of 1.5 ml 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 in a fluorescence spectrophotometer (970CRT, Sanco, China). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nmol of AMC per minute. Proteolytic activity assays were performed in duplicate and variation between duplicate samples was always <5%. The mean values were used.

2.3. Changes of potential enzyme activity in fish muscle during salting

In this paper, the enzyme activity determined under defined conditions was identified as potential enzyme activity [4], so as to differentiate it from the enzyme activity under actual conditions of salted fish processing. In order to track the changes of aminopeptidase activity from the skeletal muscle of black carp as influenced by storing with salt, approximately 200 g of pieced fish muscle (3 cm × 3 cm) were salted with 8% of sodium chloride content (w/w) at 4 °C. Fish muscle were taken on days 0, 2, 4, 6, 8, 10, and 12. In order to minimize potential contamination of aminopeptidase derived from microorganism, the surface muscle (about 1 cm × 1 cm) was cut and discarded. The inner remainder was homogenized with a three-fold volume of 25 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol (DTT) (buffer A), using a homogenizer (Kinematica, PT-2100, Switzerland) and centrifuged at 11,000 × *g* for 20 min. Then the supernatant was used to determine the aminopeptidase activity immediately as described above.

2.4. Purification of aminopeptidase

All procedures were performed under 4 °C. Approximately 360 g of minced fish muscle was homogenized with threefold volume of buffer A and centrifuged at 11,000 × *g* for 20 min in a centrifuge (Avanti J-25, Beckman). After centrifugation, the supernatant was fractionated with ammonium sulfate from 30% to 70% saturation. After centrifugation (12,000 × *g*, 20 min), the precipitate was dissolved in a minimum volume of buffer A and extensively dialyzed overnight against the same buffer. The dialyzed solution was subsequently applied to a DEAE-Sephacel anion exchange column (2.5 cm × 20 cm) previously equilibrated with buffer A containing 0.03 M NaCl. After washing the column with buffer A until the absorbance at 280 nm reached the baseline, the bound proteins were eluted with a linear gradient of NaCl from 0.03 to 0.5 M in buffer A with a total volume of 600 ml at a flow rate of 1.5 ml/min, and fractions of 5 ml were collected. Active fractions were pooled, and ammonium sulfate was added to a final concentration of 1 M and applied to a Phenyl-Sepharose 6-Fast Flow column (2.5 cm × 7 cm) pre-equilibrated with buffer A containing 1 M ammonium sulfate. The proteins retained were eluted by a decreasing linear gradient of ammonium sulfate from 1 to 0 M in buffer A in a total volume of 200 ml at a flow rate of 1.5 ml/min. Fractions showing the highest activity were further collected, dialyzed against buffer A and subsequently applied to a CHT-II hydroxyapatite cartridge (5 ml) previously equilibrated with buffer A. The enzyme was eluted using a two-step linear gradient of NaCl from 0 to 0.2 M and then to 0.4 M in buffer A in a total volume of 200 ml at a flow rate of 0.5 ml/min. Active fractions were collected for SDS-PAGE analysis and enzymatic characterization.

2.5. Protein concentration determination

The absorbance at 280 nm was used to monitor the protein eluant from chromatography columns. Protein concentrations were also determined by the method of Lowry et al. [18] with bovine serum albumin as standard.