Comparative Biochemistry and Physiology, Part B 192 (2016) 49–59

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Structure, molecular evolution, and hydrolytic specificities of largemouth bass pepsins

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ARTICLE INFO

Article history:
Received 8 August 2015
Received in revised form 16 November 2015
Accepted 20 November 2015
Available online 25 November 2015

Keywords:
Largemouth bass
Pepsin
High proteolytic activity
Thermodynamicity
Molecular evolution

ABSTRACT

The nucleotide sequences of largemouth bass pepsinogens (PG1, 2 and 3) were determined after molecular cloning of the respective cDNAs. Encoded PG1, 2 and 3 were classified as fish pepsinogens A1, A2 and C, respectively. Molecular evolutionary analyses show that vertebrate pepsinogens are classified into seven monophyletic groups, i.e. pepsinogens A, F, Y (prochymosins), C, B, and fish pepsinogens A and C. Regarding the primary structures, extensive deletion was obvious in S′1 loop residues in fish pepsin A as well as tetrapod pepsin Y. This deletion resulted in a decrease in hydrophobic residues in the S′1 site. Hydrolytic specificities of bass pepsins A1 and A2 were investigated with a pepsin substrate and its variants. Bass pepsins preferred both hydrophobic/aromatic residues and charged residues at the P′1 sites of substrates, showing the dual character of S′1 sites. Thermodynamic analyses of bass pepsin A2 showed that its activation Gibbs energy change ($\Delta G^{\ddagger}$) was lower than that of porcine pepsin A. Several sites of bass pepsin A2 moiety were found to be under positive selection, and most of them are located on the surface of the molecule, where they are involved in conformational flexibility. The broad S′1 specificity and flexible structure of bass pepsin A2 are thought to cause its high proteolytic activity.

1. Introduction

The largemouth bass (*Micropterus salmoides*) belongs to a clade of freshwater fishes endemic to North America, the part of the world that harbors more species than any other region on Earth (Briggs, 1986; Lundberg et al., 2000). Largemouth bass is the dominant top-level predator in the diverse communities of fishes in eastern North America (Henshall, 1881; Etnier and Starnes, 1993; Philipp and Ridgway, 2002). The species is expected to have strong proteolytic digestive enzymes. Previously, we purified three types of pepsinogens from largemouth bass stomach and elucidated the high proteolytic activities of bass enzymes (Miura et al., 2015).

To date, five types of tetrapod pepsinogens are known: pepsinogen A, prochymosin (pepsinogen Y), pepsinogen F, pepsinogen C (progastricsin) and pepsinogen B (Kageyama, 2002; Castro et al., 2012). In fishes, two types of pepsinogens have been classified, i.e., fish pepsinogens A and C. Recent phylogenetic trees of pepsinogens show that fish pepsinogen A is further divided into two subgroups, i.e., pepsinogens A1 and A2 (Tanji et al., 2009). Fish pepsinogen C has been shown to be monophyletic, although the zymogen gene has been duplicated at least three times in tetrapods, resulting in the functional divergence of pepsinogens BC, B, C1 and C2 (Castro et al., 2012). The occurrence of various types of pepsinogens has been shown to correlate with food habits, indicating that bovine chymosin and canine pepsin B are necessary to milk digestion (Foltmann, 1981) and gelatin digestion (Kageyama, 2006), respectively. Furthermore, some fish pepsins have been shown to have adapted to living in a cold environment to digest proteins efficiently at low temperature (Carginale et al., 2004a).

The catalytic mechanisms of known aspartic proteinases depend on the presence of two aspartate residues, namely, Asp^{32} and Asp^{215} forming an active site positioned in the middle of a molecule (Dunn, 2002). The active-site cleft accommodates a substrate, forming S4 through S′3 subsites (Powers et al., 1977; Dunn, 2002; Kageyama, 2002). The most important subsites are S1 and S′1 for the hydrolysis of a substrate. The substrate residues are usually designated as P4 though P′3, with the scissile peptide bond between P1 and P′1 (Powers et al., 1977; Dunn and Hung, 2000; Kageyama, 2002). The specificity of pepsin has been extensively investigated using small synthetic substrates, such as a pepsin substrate (KPAEF↓FRL) and its derivatives, and hydrophobic/aromatic residues have been shown to be preferred in the cleavage site (Powers et al., 1977; Dunn and Hung, 2000; Kageyama, 2006; Kageyama et al., 2010). Some types of pepsins such as bovine chymosin have been shown to have different specificities, preferring charged or polar residues at the cleavage site (Foltmann, 1981;

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http://dx.doi.org/10.1016/j.cbpb.2015.11.007
1096-4959/

Williams et al., 1997; Kageyama et al., 2010). Fish pepsins have been shown to prefer hydrophobic residues at P1 and P′1, although they also tolerate histidine and/or lysine at P1 (Brier et al., 2007). The overall digestion profiles of substrates by fish pepsins might be different from those of mammalian pepsins.

Enzyme function is strongly affected by temperature. Low temperature is associated with low levels of kinetic energy and reduced extent of molecular motions (Feller, 2010). Gastric digestion, however, does occur at low temperatures; this phenomenon is interesting from the perspective of adaptation to low temperature (Carginale et al., 2004b). Characterization of fish pepsins has shown that, although fish enzymes are labile at temperatures higher than 37 °C, they are more active than mammalian enzymes at low temperatures (Brier et al., 2007; Zhou et al., 2008; Chen et al., 2009). Previously, we have shown that bass pepsins are much more active around 20 °C than other mammalian pepsins A (Miura et al., 2015). Thus it is presumed that largemouth bass pepsin is cold-adapted, like the pepsins of arctic fish species such as Atlantic cod and Antarctic rock cod (Gildberg et al., 1990; Brier et al., 2007). Cold-adapted enzymes have shown large k_{cat} and K_m as compared with those of thermophilic and mesophilic enzymes (Yasugi et al., 2001; Suzuki et al., 2001; Fields and Houseman, 2004). Modifications of k_{cat} are due to changes in activation enthalpy, or, equivalently, in activation energy barriers to catalysis, such that cold-adapted enzymes have decreased activation Gibbs energy, and enzyme reaction can proceed through catalysis at low temperatures (Feller and Garday, 1997; Siddiqui and Cavicchioli, 2006). Some fish pepsins have been shown to be adapted to cold temperatures (Gildberg et al., 1990; Carginale et al., 2004b; Brier et al., 2007), and to have higher activity than mammalian pepsins (Tanji et al., 1988), being characterized by high k_{cat} values (Miura et al., 2015).

In the present study, we have cloned the cDNAs of largemouth bass pepsinogens and constructed phylogenetic trees. Hydrolytic specificities of bass pepsins were investigated with a pepsin substrate and its derivatives, comparing with those of porcine pepsin A. Bass pepsin A2 has dual hydrolytic specificities, preferring both hydrophobic/aromatic and charged residues; this conclusion is supported by molecular models of bass pepsins. Flexible structures have been selected positively during evolution. Thermodynamic analysis revealed lowered activation energy. These catalytic and structural adaptations are thought to cause high proteolytic activities of bass pepsins.

2. Materials and methods

2.1. Materials

Largemouth bass (*M. salmoides*) were harvested during an investigation by representatives of the Nagoya Biodiversity Center (Nagoya, Japan) into the biotic habitats of invasive aquatic carnivorous species in Tatsumaki-ike pond (Nagoya, Japan). Three types of pepsinogens, i.e., PG1, PG2 and PG3 have been purified from gastric mucosa of largemouth bass (Miura et al., 2015). They were renamed as PGA1, PGA2 and PGC, respectively, in this study. PGA1 and PGA2 have been shown to be consisting of a few subtypes. Since PGA 1-1 and PGA 2-2 were the major zymogens, they were used in the present enzymatic studies. PGC is a minor zymogen, occupying less than 5% of the total, and thus, was not used in this study. Porcine pepsinogen A was obtained from Sigma-Aldrich (St. Louis, MO, USA). RNeasy mini kit and QIAEX®II Gel Extraction kit were obtained from Qiagen (Germantown, MA, USA). High Capacity cDNA Reverse Transcription Kit was obtained from Applied Biosystems (Foster City, CA, USA). 5′/3′ RACE Kit, 2nd Generation was obtained from Roche (Mannheim, Germany). Plutinum Pfx DNA polymerase and Zero Blunt TOPO Cloning Kit were obtained from Invitrogen (Carlsbad, CA, USA). Peptides were synthesized by Sigma Genosys Japan (Ishikari, Japan). All other chemicals were of reagent grade.

2.2. Cloning and nucleotide sequences of cDNAs

Pepsinogen cDNAs of largemouth bass were obtained by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification with 3′-RACE and 5′-RACE methods. Total RNA was extracted from the largemouth bass stomach using an RNeasy mini kit. Quantity and quality of the RNA were assessed by OD_{260}/OD_{280} and agarose gel electrophoresis. Two micrograms of total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit. 5′-RACE and 3′-RACE primers were constructed based on known sequences of fish pepsinogen genes. The products of cDNAs for bass pepsinogens were amplified in a thermocycler using Plutinum Pfx DNA polymerase and designed primers in a total volume of 50 μl according to the following schedule: 5 min at 94 °C; 27 cycles each consisting of 1 min at 94 °C, 1 min at 45 °C, and 1 min at 68 °C; 5 min at 68 °C. Amplification products were resolved on a 1.2% agarose gel using the 100 bp DNA ladder (BioNEER, Daejeon, Korea) for markers and purified using a QIAEX®II Gel Extraction kit.

Purified cDNA products were ligated into the pCR bluntII-TOPO vector using a Zero Blunt TOPO Cloning Kit. Recombinant plasmids were transformed into DH5α competent cells. Colonies containing the insert were screened on 1.2% agarose gels and sequenced with an ABI PRISM 3500xl Genetic Analyzer.

2.3. Molecular modeling of largemouth bass pepsins

Tertiary structural models of bass pepsins A1 and A2 and their complex with peptide substrates were constructed with the program Modeller 9.12 (Sali and Blundell, 1993) using as templates the tertiary structures of human (PDB code; 3UTL) (Bailey et al., 2012), porcine (PDB code; 5PEP) (Andreeva et al., 1984; Cooper et al., 1990; Sielecki et al., 1990), and Atlantic cod (PDB code; 1AM5) (Karlsen et al., 1998) pepsins A and the complex between human pepsin A and a synthetic phosphonate inhibitor (Fujinaga et al., 2000).

2.4. Molecular phylogenetic analysis

The alignment of the amino acid sequences of various animal pepsinogens including bass zymogens was assessed using the Probcons software program (Do et al., 2005). This step enabled us to apply deduction of the alignment of cDNA nucleotide sequences. A phylogenetic tree based on nucleotide sequences were constructed according to the Bayesian method in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003). The sequence divergence was calculated among all pairwise comparisons following the GTR (Tavare, 1986). Robustness of each node in the phylogenetic tree was assessed by posterior probability based on 20,000 generation. The likelihood ratio tests of synonymous and non-synonymous nucleotide substitutions for detecting positive selection were carried out according to the methods of Yang (1998) and using PAML software 4.7a (Yang, 2007).

2.5. Assay of peptide hydrolyzing activity

Peptide hydrolyzing activity was measured using purified largemouth bass pepsinogens after activation. The reaction mixture contained 0.5 M sodium formate buffer, pH 4.0, 50 μM of peptide (KPAEF**X**RL, KPA**G**F**X**RL and KPA**X**FFRL), and an appropriate amount of activated bass enzyme. The total volume was 20 μl. After incubation at 20 °C for 30 min, the reaction was stopped by the addition of 80 μl 3% perchloric acid (PCA). Following the removal of any precipitated material by centrifugation at 12,000 rpm for 2 min, reaction mixtures were subjected to HPLC on a column of TSKgel ODS-120 T (TOSOH, Tokyo, Japan) that had been equilibrated with 0.1% trifluoroacetic acid (TFA). Data are presented as the mean ± SD. The statistical significance was compared between three groups by means of one-way ANOVA and