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Purification and characterization of a novel imidazole dipeptide synthase from the muscle of the Japanese eel *Anguilla japonica*

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Abstract

We have purified a novel enzyme from eel white muscle which catalyzes the syntheses of imidazole dipeptides, such as carnosine (β-alanyl-L-histidine), anserine (β-alanyl-π-methyl-L-histidine), and balenine (ophidine; β-alanyl-τ-methyl-L-histidine), directly from their precursors. The enzyme was purified 1130-fold from eel muscle by a series of column chromatographies. Although eel muscle contains a large amount of carnosine and only trace amounts of anserine and balenine, the anserine synthesizing activity was by far the highest. From gel permeation chromatography, the molecular mass of the enzyme was calculated to be 275 kDa. SDS-PAGE of the purified enzyme represented a band around 43 kDa, suggesting that the native enzyme is a hexamer or heptamer. The optimal pH and temperature were around 9.5 and 60 °C, respectively. K_m values for β-alanine and π-methyl-L-histidine were 44 and 89 mM, respectively. The enzyme was greatly activated by Zn^{2+} and inhibited by EDTA. The N-terminal amino acid sequence of 25 residues of the purified enzyme showed 52% amino acid identity to 38–62 residues of zebrafish haptoglobin precursor. The purified enzyme also exhibited hydrolytic activity against these imidazole dipeptides.

Keywords: Imidazole dipeptides; Carnosine; Anserine; Balenine; Imidazole dipeptide synthase; Japanese eel; Muscle; *Anguilla japonica*

1. Introduction

Imidazole dipeptides in vertebrates, i.e., carnosine (β-alanyl-L-histidine), anserine (β-alanyl-π-methyl-L-histidine), and balenine (ophidine; β-alanyl-τ-methyl-L-histidine), have long been known to exist in excitable tissues such as skeletal muscle and brain, and are proposed to have versatile physiological functions. Of these dipeptides, carnosine received special attention as a research target for functional analyses because of the availability of the reagent. Carnosine has been proven to work as a proton buffering constituent in fast-twitch white muscle (Abe et al., 1985; Abe and Okuma, 1991; Abe, 2000), as an antioxidant and scavenger for reactive oxygen species (Kohen et al., 1988; Boldyrev et al., 1989, 2004; Boldyrev and Abe, 1999), as a neurotransmitter or modulator (Margolis, 1974; Bonfanti et al., 1999), as a neuroprotector (Hipkiss et al., 1997; Boldyrev et al., 2004), and as an anti-aging agent (Hipkiss, 2000; Hipkiss et al., 2001).

A large amount of carnosine is found in human muscle (10 mM) and in sprinting mammals such as horses (34 mM) and oxen (20 mM) (Abe, 1995). Although the distribution of carnosine in fish muscle is rather restricted, a high concentration of carnosine is resident in eel muscle (20 mM). Anserine, on the other hand, is abundant in chicken pectoral muscle (43 mM) and the white muscle of fast swimming fish such as tunas (27 mM), skipjack tuna (51 mM), and blue marlin (105 mM). Copious concentrations of balenine is found only in whales (78 mM) and snakes (13 mM), although balenine has been found in trace amounts of all vertebrate muscles thus far examined (Abe, 1995). Apparently, the distribution of these imidazole dipeptides in vertebrate muscle is highly species specific and does not have any phylogenetic relationship.

Although many researchers have focused on the physiological functions, information on the biosynthetic pathway and the constituent enzymes that catalyze formation of these dipeptides has remained deficient. Carnosine is known to be synthesized from β-alanine and L-histidine by carnosine synthetase (EC 6.3.2.11) in the presence of ATP, which has been characterized from chick pectoral muscle (Kalyankar and Meister, 1959;

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Winnick and Winnick, 1959, 1960; Stenesh and Winnick, 1960; Bauer and Schulz, 1994), mouse olfactory system (Harding and Margolis, 1976), and rat and mouse brains (Skaper et al., 1973; Bauer et al., 1979, 1982). No information, however, has been published on the primary structure of carnosine synthetase.

Anserine, on the other hand, has been proposed to be synthesized by two different pathways. The first is the direct methylation of carnosine catalyzed by carnosine-*N*-methyltransferase (EC 2.1.1. 22) in the presence of S-adenosyl-L-methionine, which was confirmed in chick pectoral muscle (McManus, 1962; Bauer and Schulz, 1994). The second pathway is the ATP-dependent condensation of the precursors, β-alanine and π-methyl-L-histidine, by carnosine synthetase or some closely related enzyme (Winnick and Winnick, 1959, 1960; Stenesh and Winnick, 1960).

In contrast to the synthesis of imidazole dipeptides, their hydrolysis has been elucidated in more detail. Carnosine is hydrolyzed by carnosinases (Rosenberg, 1960; Harding and Margolis, 1976; Lenney, 1976) which are classified into two groups, tissue carnosinase (cytosolic non-specific dipeptidase; EC 3. 4.13.18) and serum carnosinase (EC 3. 4.13. 20), both of which belong to a large family of metalloproteases. Recently, cDNAs of human brain carnosinase and cytosolic non-specific dipeptidase were cloned and characterized (Teufel et al., 2003). Moreover, an anserine hydrolyzing enzyme, anserinase (EC 3. 4.13. 5), was purified from the brain of Nile tilapia *Oreochromis niloticus* and its nucleotide and amino acid sequences were identified (Yamada et al., 2005).

For these enigmatic imidazole dipeptides, their physiological functions and synthetic pathways, as well as the reasons for their species specific distribution remain unsolved. In this report, we describe the purification and characterization of a novel imidazole dipeptide synthase from the muscle of the Japanese eel *Anguilla japonica*, which catalyzes both the synthesis and hydrolysis of these three dipeptides.

2. Materials and methods

2.1. Materials

Live Japanese eel (*Anguilla japonica*) were obtained from the Tokyo Metropolitan Central Wholesale Market (Tokyo, Japan). β-Alanine was purchased from Wako Pure Chemical Industries (Osaka, Japan). L-Histidine, π-methyl-L-histidine (1-methyl-L-histidine), carnosine, and γ-aminobutyric acid were from Sigma-Aldrich (St. Louis, MO, USA). τ-Methyl-L-histidine (3-methyl-L-histidine) was obtained from Bachem (Bubendorf, Switzerland). Anserine and balenine were prepared from the skeletal muscle of big-eye tuna *Thunnus obesus* and minke whale *Balaenoptera acutorostrata*, respectively (Abe and Okuma, 1991). TSK-gel DEAE-Toyopearl 650M and Ether-Toyoperl 650M were obtained from Tosoh (Tokyo, Japan). His-Trap HP, MonoQ HR5/5, and Superdex 200 10/300 were obtained from Amersham Biosciences (Uppsala, Sweden). Macro-prep Ceramic Hydroxyapatite Type 1 (CHT-1) column was from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade and purchased either from Sigma-Aldrich or Wako unless otherwise stated.

2.2. Assay of imidazole dipeptide synthetic activities

Dipeptide synthetic activities were assayed by measuring the formation of products by high performance liquid chromatography (HPLC) according to the method of Togashi et al. (1998) with some modifications. The standard incubation mixture for the assay of anserine synthesis consisted of 160 mM π-methyl-L-histidine, 250 mM β-alanine, and 2 mM ZnCl$_2$ in 50 mM Tris–HCl, pH 7.5. For carnosine or balenine synthesis, L-histidine or τ-methyl-L-histidine was used, respectively, instead of π-methyl-L-histidine. γ-Aminobutyric acid and L-histidine were used as substrates for homocarnosine synthesis. The enzyme preparation was added to the reagent mixture prepared on ice and incubated for 1 h at 37 °C unless otherwise described. The reaction was stopped by placing the tubes in a boiling water bath for 3 min followed by centrifugation at 2300 ×*g* for 3 min. The supernatant was filtered with Ultrafree-MC (Millipore; Bedford, MA, USA) and used for HPLC analysis. For a time course experiment, aliquots of reaction mixture were taken out at appropriate time intervals (0 to 24 h) and treated as above.

The HPLC determination of dipeptides was carried out on a Jasco HPLC system (Jasco; Tokyo, Japan) with a Superiorex ODS column ($^{\varphi}$4.6 × 250 mm, Shiseido; Tokyo, Japan). Elution was performed isocratically with 50 mM KH$_2$PO$_4$, pH 3.4, containing 6 mM 1-heptanesulfonic acid and 2% acetonitrile at a flow rate of 1 mL/min at 50 °C and monitored at 210 nm.

Protein concentration was measured with a protein assay dye solution (Bio-Rad Laboratories) using bovine serum albumin as a standard. During enzyme purification, protein elution was monitored by the absorbance at 280 nm using a micro plate reader, SpectraMax M2 (Molecular Devices; Sunnyvale, CA, USA).

2.3. Purification of imidazole dipeptide synthase

During the purification, the enzyme activity was checked for anserine synthesis. All purification procedures were performed at 4 °C. The white muscle of Japanese eel, weighing about 400 g, was homogenized with 5 vol of 50 mM Tris–HCl, pH 7.5, and the homogenate was centrifuged at 12,000 ×*g* for 15 min.

Ammonium sulfate was added to the supernatant up to 50% saturation. After being stirred for 2 h, the suspension was centrifuged as above and the supernatant was brought to 75% saturation with ammonium sulfate. The resultant precipitate (50–75% fraction) was dialyzed overnight against 10 mM Tris–HCl, pH 8.0. After centrifugation as above, the supernatant was applied onto a DEAE-Toyopearl column (3 × 50 cm) previously equilibrated with the same buffer, and the column was washed with 500 mL of the same buffer. The enzyme was eluted with a linear gradient of 0−250 mM NaCl at a flow rate of 2 mL/min. The active fractions were collected and ammonium sulfate was added to the solution at a final concentration of 2 M.

This pooled solution was loaded onto an Ether-Toyopearl column (3 × 15 cm) equilibrated with the same buffer containing 2 M ammonium sulfate, and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of 2–0 M ammonium sulfate in the same buffer at a flow rate of 2 mL/min.