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Purification and characterisation of an alanine aminopeptidase from bovine skeletal muscle

Xiu Juan Ye, Tzi Bun Ng *

School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

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ABSTRACT

A monomeric alanine aminopeptidase was purified to a single band in SDS–PAGE from bovine skeletal muscle by using a procedure including ammonium sulphate fractionation, adsorption on DEAE–cellulose, gel filtration on Ultrogel ACA 34, and adsorption on hydroxyapatite. Molecular weight determination by gel filtration and sodium dodecyl sulphate–polyacrylamide gel electrophoresis yielded a molecular size of 60 kDa. The aminopeptidase activity was optimal at pH 8.0 and 37 °C. It was totally abolished by Co^{2+} and Zn^{2+} ions, and almost completely inhibited by bestatin and Mn^{2+}. The activity was strongly inactivated by phenylmethansulfonyl fluoride, Mg^{2+}, and Fe^{3+} ions but stimulated by pepstatin and EDTA. However the activity was not affected by Ca^{2+}, puromycin and iodoacetate. When compared with its activity toward Ala-β-naphthylamides (Ala-βNA), the enzyme exhibited 15–17% as much activity toward Pro- and Leu-βNA, 4–6% activity toward Met- and Arg-βNA, and negligible or no activity toward Glu- and Ser-βNA.

1. Introduction

Aminopeptidases are found in animals, plants, and microorganisms (Barrett, Rawlings, & Woessner, 1998). These enzymes are detected in the cell membrane, the cytoplasm and organelles (Taylor, 1993). They play a role in metabolism of hormones neurotransmission, cell maturation, utilisation of exogenous proteins, and removal of nonfunctional proteins (Gonzales & Robert-Baudouy, 1996).

Aminopeptidases have been reported from a variety of animal tissues including the brain (Hayashi & Oshima, 1980), lungs (Simmons & Orawski, 1992), kidney (Wachsmuth, Fritze, & Pfleiderer, 1966), liver (Kawata, Imamura, Ninomiya, & Makisumi, 1982), small intestine (Hasegawa, Kodama, & Akatsuka, 1985), and muscle (Otsuka, Okitani, Katakai, & Fujimaki, 1976). The tissues from which aminopeptidase were isolated originated from a number of animals including human (McClennan & Garner, 1980), pig (Flores, Aristoy, & Toldrá, 1998), rabbit (Matsuishi, Saito, Okitani, & Kato, 2003), chicken (Hui et al., 1993; Jamadar, Jamadar, Dandekar, & Harikumar 2006; Skrtic & Vitale, 1994), carp (Hara, Sakai, & Ishihara, 1988; Liu et al., 2008), red sea bream (Wu, Cao, Chen, Liu, & Su, 2008), and mullet (Chiou, Matsui, & Konosu, 1988). Many proteins are present in muscle, including cathepsins, calpains, dipeptidyl peptidases and aminopeptidases. Aminopeptidases are involved in the production of free amino acids in meat and meat products and this contributes to an improvement of meat flavour (Flores, Marina, & Toldrá, 2000). Different kinds of muscle aminopeptidases comprising human pyroglutamyl and leucine aminopeptidases, bovine aminopeptidase D and H, porcine methionyl aminopeptidases and chicken aminopeptidase H (Flores et al., 2000; Mantle, Lauart, & Gibson, 1991; Nishimura, Rhyu, Kato, & Arai, 1994; Rhyu, Nishimura, Kato, Okitani, & Kato, 1992), red sea bream (Wu et al., 2008) and carp leucine aminopeptidases (Liu et al., 2008) have been reported. The aim of the present investigation was to isolate an aminopeptidase from bovine skeletal muscle and to compare its characteristics with other aminopeptidases reported in the literature.

2. Materials and methods

2.1. Materials

After slaughter, the fresh bovine skeletal muscle was stored at 0 °C. Before the experiment, the sample was exposed to 3–4 °C for 4 days. DEAE–cellulose, Ultrogel ACA 34, and Hydroxyapatite were from Whatman. Ala-βNA (βNA: β-naphthylamides), Arg-βNA, Glu-βNA, Leu-βNA, Met-βNA, Pro-βNA, and Ser-βNA were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Phenylmethansulfonyl fluoride (PMSF), pepstatin, puromycin, bestatin, sodium iodoacetated (ICH_2COONa), and ethylendiaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co.

2.1.1. Assay of aminopeptidase activity

Sample containing aminopeptidase was incubated with 0.5 mM Ala-βNA as substrate in 0.4 ml of 50 mM. Tris–HCl buffer (pH 7.2) at 37 °C for 20 min. Then 0.4 ml of 0.23 N HCl in ethanol and 0.4 ml

* Corresponding author. Tel.: +86 852 2609 6872; fax: +86 852 2603 5123.
E-mail address: b021770@mailserv.cuhk.edu.hk (T.B. Ng).

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of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol were added to the reaction mixture in order to stop the enzyme reaction. The amount of βNA formed was measured at 540 mm.

The activity of the enzyme toward other substrates including Arg-βNA, Glu-βNA, Leu-βNA, Met-βNA, Pro-βNA, and Ser-βNA was similarly determined.

2.1.2. Determination of protein concentration

The concentration of protein was determined according to the Folin–Lowry method, using bovine serum albumin as the standard.

2.1.3. Purification of an alanine aminopeptidase from bovine muscle

All steps were carried out at about 4 °C.

2.1.3.1. Extraction and ammonium sulphate fractionation. Bovine skeletal muscle (197 g) was homogenised in 600 ml of 40 mM Tris–HCl buffer (pH 8) containing 0.1% 2-mercaptoethanol with a Waring blender gently for 15 s and vigorously for 45 s. The homogenate was then centrifuged at 7500 rpm for 20 min and the supernatant was filtered through three layers of gauze to remove floating fat. The filtrate was subjected to fractionation with 0–80% saturated ammonium sulphate. The mixture was centrifuged at 7500 rpm for 20 min, and the precipitate collected was dissolved in and dialysed against 10 mM Tris–HCl buffer (pH 7.2).

2.1.3.2. DEAE–cellulose column chromatography. A DEAE–cellulose column (3.25 cm × 23 cm) was equilibrated with 10 mM Tris–HCl buffer (pH 7.2) containing 0.1% 2-mercaptoethanol. The sample from the previous step was applied to the column. After the column had been washed with three volumes of equilibration buffer to completely remove unabsorbed protein, the adsorbed protein was eluted at a flow rate of 120 ml/h with a linear gradient of 0–0.24 M NaCl in the same buffer. Fractions of 8 ml were collected and tested for Ala-βNA hydrolysing activity.

2.1.3.3. Ultrogel ACA 34 column chromatography. Fractions with enzyme activity from the DEAE–cellulose column were concentrated by ultrafiltration, and subsequently applied on an Ultrogel ACA34 column (3.14 cm × 11.2 cm) which had been equilibrated with 100 mM NaCl in 10 mM Tris–HCl buffer (pH 7.2) containing 0.1% 2-mercaptoethanol. The enzyme was eluted with the same buffer at a flow rate of 100 ml/h. Fractions of 5 ml were collected and tested for Ala-βNA hydrolysing activity.

2.1.3.4. Hydroxylapatite column chromatography. The fraction with aminopeptidase activity from the previous step was dialysed against 10 mM potassium–phosphate buffer (pH 7.2) containing 0.1% 2-mercaptoethanol, and was subsequently applied onto a hydroxylapatite column (3.14 cm × 12 cm) already equilibrated with the same buffer. Fractions were eluted at a flow rate of 120 ml/h with a linear gradient of 10 mM to 300 mM potassium–phosphate buffer. Fractions of 4 ml were collected and tested for Ala-βNA hydrolysing activity.

2.1.3.5. SDS–PAGE. Gel electrophoresis was performed following the method of Laemmli using 12% SDS, and bromophenol blue was used as the tracking dye. After gel electrophoresis the proteins were stained with Coomassie blue.

2.1.4. Optimum temperature and pH of enzyme

Optimum temperature was determined by incubating the isolated enzyme with 0.5 mM Arg-βNA in 50 mM Tris–HCl buffer (pH 7.2) for 20 min, at various temperatures ranging from 0 °C to 80 °C, in a total reaction volume of 400 μl. OD_{540} was taken as an index of enzyme activity. One unit of enzyme is the amount of enzyme that causes the release of Ala-βNA followed by an OD increase of 1 per min at 540 nm.

Optimum pH was determined by incubation of the isolated enzyme with 0.5 mM Ala-βNA at 37 °C for 20 min, in a total volume of 400 μl using the following buffers spanning the pH range of 2.68–10: 100 mM acetate buffer (pH 2.68–5.39); 100 mM phosphate buffer (pH 4.31–8); and 100 mM ammonia buffer (pH 7.4–10.29). The increase in OD_{540} was recorded. The highest enzyme activity corresponded to 100% enzyme activity.

2.1.5. Effects of possible inhibitors and cations on enzyme activity

In a 400 μl reaction mixture in 50 mM Tris–HCl buffer (pH 7.2), the enzyme was preincubated in the presence or absence of various possible inhibitors at the final concentration: 10 mM EDTA, 10 mM ICH_2OONa, 10 mM PMSF, 0.1 mM pepstatin, 0.1 mM purimycin, 0.1 mM bestatin and the following cations at a final concentration of 1 mM: Mg^{2+}, Fe^{3+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} for 30 min at 37 °C. The rate of hydrolysis of Arg-βNA was measured. Enzyme activity in the absence of any inhibitor and cation was set at 100%.

2.1.6. Statistical analysis

The data were analysed by analysis of variance ANOVA followed by Duncan's multiple range test. A p value of less than 0.05 indicated statistically significant difference.

3. Results

3.1. Purification of alanine aminopeptidase from bovine skeletal muscle

Alanine aminopeptidase activity was detected in the second absorbance peak (D2) which was adsorbed on the DEAE–cellulose column (Fig. 1A), and subsequently in the second absorbance peak (U2) from the Ultrogel ACA 34 column (Fig. 1B). In the last purification step, activity was detected in the largest absorbance peak (H2). It appeared as a single 60-kDa protein in SDS–PAGE (Fig. 2). Alanine aminopeptidase was purified 114-fold from the crude extract, and after 3 steps of purification. The recovery of activity was 4% (Table 1).

3.2. Effects of temperature and pH on aminopeptidase activity

Optimal activity was found at 37 °C. Alanine aminopeptidase activity was relatively stable until 55 °C (Fig. 3A). However, the activity underwent an abrupt decline when the temperature was elevated from 60 °C to 80 °C (Fig. 3A). The enzyme activity increased progressively when the pH was raised from 5.39 to 8. The optimum pH was 8. When the pH was increased the activity plummeted abruptly until it was indiscernible at pH 10.3 (Fig. 3B).

3.3. Effect of inhibitors and cations on purified alanine aminopeptidase

The isolated alanine aminopeptidase was completely inhibited by Co^{2+} and Zn^{2+} ions (Fig. 4A). It was destroyed to a large extent (about 70%) by PMSF, Mn^{2+}, and Fe^{3+} ions (Fig. 4A and B). The activity was nearly (95–98%) abrogated in the presence of bestatin and Mn^{2+} (Fig. 4A), but enhanced by 25–35% in the presence of pepstatin and EDTA (Fig. 4B).

3.4. Substrate specificity of purified alanine aminopeptidase

The purified aminopeptidase exhibited the highest activity toward Ala-βNA. Its activity toward Leu-βNA and Pro-βNA was much lower, being (16.8 ± 2.3)% and (15.1 ± 2.1)% (mean ± SD, n = 3). The aminopeptidase expressed minimal activity toward Arg-βNA, and