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Effects of Chelators and Metal Ions on purified Leucine-specific Aminopeptidase from *Aeromonas caviae* T-58

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Abstract

An extracellular aminopeptidase (AMP) of a bacterial soil isolate, Aeromonas caviae T-58, was purified to electrophoretically homogeneity by ammonium sulfate precipitation and ion-exchange chromatography (Q-Sepharose fast flow and Mono-Q column) to 48-fold with a yield of 3.0%. The purified native enzyme is a monomer and exhibited a single band with molecular weight of 32 kDa estimated by SDS/polyacrylamide-gel electrophoresis. The enzyme was inactivated by Mn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}, but not affected by Ca^{2+}, Ba^{2+}, Zn^{2+}, Al^{3+}, Ni^{2+}, Li^{2+}, Pb^{2+}, and Mg^{2+}. EDTA completely inhibited enzyme activity indicative of the enzyme to a metalloenzyme type. The addition of 1 mM Zn^{2+} restored 100% activity of EDTA-inhibited enzyme while 1 mM Co^{2+} restored 10% activity. However, the addition of equimolar concentrations of both metals showed a non co-catalytic effect as residual activity reduced to 90%. The enzyme therefore possibly belongs to a catalytic family of Zn^{2+} metalloenzyme and does not require Ca^{2+} for enzymatic activation. The purified enzyme showed a high affinity for L-Leu- p-nitroanilide and valine but not with proline, glycine or alanine-pNA.

Introduction

The addition of specific proteolytic enzymes accelerates the maturation steps involved in the formation of peptides and amino acids in the functionality and aging of foods. Aminopeptidases are proteolytic enzymes that catalyze the removal of amino acid residues at the N-terminal position of peptides and proteins. Aminopeptidases are important in the fermentation of yogurt and ripening of cheese (Miyakawa *et al.*, 1992; Raksakulthai *et al.*, 2002). They also play significant roles in biological processes, such as protein maturation, hormone production, and peptide digestion (Gonzales and Robert-Baudouy, 1996). The use of modified starter, semi-liquid slurry to shorten the maturation period of cheese is fraught with problems, which include high starters cost, non-reproducibility of results, and long period to develop desired flavor or texture. Aminopeptidases have been purified from many sources such as *Lactobacillus helveticus* (Miyakawa *et al.*, 1992)) *Aeromonas caviae* (Izawa *et al., 1997*), *Grifola frondosa* (Nishiwaki and Hayashi, 2001) and *Illex illecebrocus* (Raksakulthai *et al.*, 2002).

Materials and Methods

Microorganism and culture conditions

The *Aeromonas caviae* T-58 used in this study was previously isolated from a soil sample at the National Food Research Institute, Tsukuba. The organism was cultivated aerobically at 30°C for 24 h on a rotary incubator at a speed of 200 rpm in 500-mL Erlenmeyer flask containing 100 mL

rotary incubator at a speed of 200 rpm in 500-mL Erlenmeyer flask containing 100 mL nutrient medium (pH 7.0); tryptone 1% (w/v), glucose 0.1% (w/v), Bacto-soy 0.1% (w/v), yeast extract 0.1% (w/v), K_2HPO_4 0.1% (w/v), NaCl 0.1 %(w/v), and $MgSO_4.7H_2O$ 0.01 %(w/v).

Aminopeptidase activity

Aminopeptidase activity was assayed by measuring the amount of *p*-nitroaniline released in a reaction mixture consisting of 100 µL 2 *mM* Leucine-*p*NA (Nacalai Tesque Inc., Kyoto, Japan) as a substrate, 125 µL 50 *mM* Tris-HCl (pH 8.0), 225 µL H_2O and 50 µL of enzyme solution incubated at 30°C for 10 min. The reaction terminated by adding 500 µL of 1N acetic acid and the absorbance measured at 405 nm using a Beckman DU® 640 spectrophotometer.

Purification of Aminopeptidase

The enzyme was purified in five steps namely; Step 1: Preparation of cell-free extract, Step 2: Ammonium sulfate precipitation, Step 3: Chromatography on Q Sepharose Fast flow column (XK 26/10), *Step 4: Chromatography on Q-Sepharose Fast flow column (Hiload 16/10)* and Step 5 via Mono-Q HR 5/5 (Amersham Pharmacia Biotech).

Determination of Molecular Weight

The purified enzyme was subjected to SDS-PAGE on 1 *mM* thick 12% acrylamide gel with Tris-glycine (pH 8.3) as electrode buffer. The samples were dissolved in a Tris-HCl loading buffer containing 1% (w/v) SDS, 20% (v/v) glycerol and 2% (v/v) 2-mercaptoethanol, and heated in boiling water for 3 min according to the method of Laemmli (1970). After electrophoresis gel was stained with 0.25% solution of Coomassie Brilliant Blue (CBB) R-250 (Wako pure chemical industry, Osaka) and the excess dye was washed out using 10% acetic acid on a shaker. The molecular mass and homogeneity of the enzyme was estimated using molecular weight marker with the range of 10-220 kDa (molecular weight marker DAIICHI, Daiichi pure chemicals).

Specific activity

One unit of enzyme activity is the amount of enzyme required to liberate one µmole of *p*-nitroaniline per minute at 30°C (pH 8.0). Protein was determined by the method of Bradford (1976) (Bio-Rad, Hercules, USA) using Bovine serum albumin as standard and absorbance read at 280 nm.

Effect of chelators and Metal Ions

The enzyme was treated with 5*mM* EDTA by incubating in 50 *mM* 2-[*N*-morpholino] ethanesulfonic acid (MES) (pH 6.5) and 50 *mM* Tris-HCl (pH 8.0) at 30°C for 30 min respectively and residual activity determined under standard assay condition. In a separate study, the purified enzyme (10 µg/ml) was dissolved in 50 *mM* Tris-HCl buffer (pH 8.0) and pre-incubated with 1 *mM* metal salts and 1 mM EDTA at 30°C for 30 min followed by mixing 450 µml pre-incubated substrate solution. Residual enzyme activity was then assayed under standard conditions. The ability of metal salts to restore enzyme activity of a completely EDTA-inactivated enzyme was done by treating EDTA-inactivated enzyme with 1 *mM* $CaCl_2$, $ZnCl_2$, $FeSO_4$, $AlCl_3$, $MnCl_2$, $LiCl_2$, $NiCl_2$, $PbCl_2$, $BaCl_2$, $CoCl_2$, $MgCl_2$, $CuCl_2$, and $CdCl_2$ and restored activity determined in a standard assay condition.

Results and Discussion

Purification of Aminopeptidase

A summary of the purification processes showing the specific aminopeptidase activity observed with Leu-*p*NA as substrate is shown in Table 1. The detection of a single band on SDS-PAGE demonstrated the purity of the aminopeptidase and confirmed by gel filtration to be a monomer of 32 kDa (Figure 1). Several authors have reported different molecular weight values for leucine aminopeptidases and these values depend on sources and number of subunits.

Table 1: Summary of purification of aminopeptidase from *A. caviae* T-58

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	307.5	825	2.7	100	1
90% $(NH_4)_2SO_4$ fraction	142.4	652.5	4.6	79	1.7
First Q-Sepharose	9.06	243	26.8	29.5	10
Second Q-Sepharose	1.26	65.3	52	7.9	19.4
Mono Q	0.21	26.6	127.5	3.2	47.5

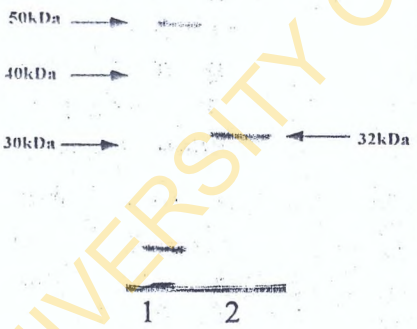

Fig.1. SDS-PAGE of the purified aminopeptidase from *Aeromonas caviae* T-58 The enzyme was subjected to SDS-PAGE in a 12% (w/v) polyacrylamide slab gel at pH 8.3. Protein was detected by Coommassie Brill int blue R-250 staining. Lanes: 1, standard; 2 purified aminopeptidase.

Activation and inhibition by metal ions

The purified native enzyme was inhibited by Mn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}, but not affected by Ca^{2+}, Ba^{2+}, Zn^{2+}, Al^{2+}, Ni^{2+}, Li^{2+}, Pb^{2+}, and Mg^{2+}. Izawa *et al.*, 1997 reported a similar observation when an aminopeptidase was treated under similar conditions while Osada and Isono, 1986) had reported aminopeptidase from *Xanthomonas citris* to be Mn^{2+} and Mg^{2+} activated but inhibited by Cu^{2+}, Zn^{2+} and Cd^{2+}. A report by Miyakawa *et al.* 2001 however differed in that aminopeptidase from *Lactobacillus helveticus* was strongly activated by the addition of 1 *mM* Co^{2+}. On the other hand, report by Nishiwaki and Hayashi (2001) indicated restoration of activity in the order of 68, 58, 34 and 6% when EDTA-treated aminopeptidase from edible basidiomycete *Grifola frondosa* was treated with 0.03 *mM*

Cu^{2+}, Zn^{2+}, Co^{2+} or Mn^{2+} respectively. Addition of 1 *mM* EDTA did not inhibit but increased enzyme activity at pH 8.0, but at pH 6.5 it inhibited the enzyme activity over a period of 24 h. In this case, the EDTA - inactivated enzyme was stable up to 72 h at 4°C as 100% activity was restored by the addition of 1 mM Zn^{2+}, and about 50% activity retained two months in storage under the same conditions. The role of Co^{2+} in the catalysis of the enzyme in this study is somewhat ambiguous as the addition of 1 *mM* Co^{2+} in the native enzyme inhibited catalysis while the addition of the same amount of Co^{2+} to the EDTA-inactivated enzyme restored 10% activity. However, addition of same amount of Zn^{2+} restored 100% of the original activity. On the other hand, the addition of equimolar concentrations of Zn^{2+} and Co^{2+} lowered residual activity to 90%. Zn^{2+} may possibly be the natural co-factor needed for physiological activity of this enzyme since 100% activity was restored even in the absence of Co^{2+}. This implies that Zn^{2+} and Co^{2+} may therefore not play a supportive role in the co-catalysis of the enzyme, and that Co^{2+} likely plays a modulating role in the presence of Zn^{2+}. The removal of Zn^{2+} resulted in loss of activity, whereas replacement of the metal up to 1 *mM* resulted in a quantitative restoration of activity. The observation is contrary when Zn^{2+} replaced Co^{2+}. The removal of the native zinc ion by the chelator and dialysis for 1h, and t e subsequent restoration of activity by the addition of 1 *mM* Zn^{2+} is an indication of the metal-binding characteristics of the chelator and not by its hydrophobic or ionic properties. It was observed that the addition of Zn^{2+} above 1 *mM* to EDTA-inhibited enzyme resulted in loss of activity indicating that the Zn^{2+} requirement for the enzyme is about 1 *mM*. This enzyme is therefore a metalloenzyme, which is in consonant with other reported aminopeptidases in this group.

Conclusion

Extracellular aminopeptidase enzyme (AMP) was isolated and purified to an electrophoretically pure state from a bacterial soil-isolate *Aeromonas caviae* T-58. This enzyme had higher specificity towards L-leu-*p*NA when compared with other *p*NA substrates investigated. The enzyme requires Zn^{2+} to activate its catalytic domains for maximal activity, and it is stable for several days in the presence of this metal, but it is rapidly inactivated by presence of EDTA.

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