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The pH and thermal response of purified leucine-specific aminopeptidase from *Aeromonas caviae*

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Abstract
A purified extracellular monomeric leucine-specific aminopeptidase from *Aeromonas caviae* T-58 with molecular mass of 32 kDa was subjected to varying pH and temperature conditions to determine its response. The activity of the enzyme was maximal at 65°C but stable up to 40°C. Optimum pH was 8.0 and pH stability had a range of 6 and 10.

Introduction

Enzymes are bio-catalysts that accelerate chemical reactions and do not in themselves involved and can be recovered that the end of the reaction. Enzymes function within specific pH and thermal limits which determine their functionality in cellular metabolisms. pH dramatically affects the ionization, and hence the molecular conformation and biological activity of enzymes and hence their biochemical functionality. Enzymes are therefore classified as acidic, basic or neutral in the activity. Aminopeptidases are exopeptidic enzymes that catalyze the removal of amino acid residues at the N termini of peptides and proteins. The functionality and character of aminopeptidases generally depend upon their substrate specificities, such as preference for a neutral, acidic, or basic amino acid in the P1 position of the amino terminus of peptides (Wilk *et. al.*, 1998). Aminopeptidases from different organisms have shown a wide range of values with respect to their responses to pH and temperature. It is therefore imperative to determine the reaction of aminopeptidase from *Aeromonas caviae* T-58 to pH and temperature variations.

Materials and Methods

Microorganism and culture conditions

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 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 (*p*NA) 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 sulphate 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).

Effects of temperature and pH on Aminopeptidase (AMP)

The effect of pH on aminopeptidase activity was determined in the range from pH 2.2 to 11.5 by using the following using 50 *mM* each of buffers; sodium citrate (pH 2.2- 4.2), sodium acetate (pH 3.4 - 5.7), 2-[*N*-Morpholino]ethanesulphonic acid (MES) (pH 5.1-7.2), 3-[N-Morpholino]-propanesulfonic acid (MOPS) (pH 6.4-8.2), *N*-2-

[Hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) (pH 6.6-8.6), *N*-Tris[hydroxymethyl]-methylglycine (pH 7.1-8.9), 2-(N-cyclohexyl-amino) ethanesulfonic acid (CHES) (pH 8.1-9.4), 3-[cyclohexylamino]propanesulfonic acid (CAPS) (pH 8.6-11.5). To estimate the thermal stability of the enzyme, the enzyme was pre-incubated in 50 *mM* Tris-HCl buffer (pH 8.0) for 30 min at a range of 0-100°C and the remaining activity measured by standard assay. To estimate the pH stability of enzyme, the enzyme was pre-incubated with the same buffer conditions described earlier (pH 2.2-11.5) at 30°C for 30 min followed by determination of residual activity in a standard assay condition. The influence of pH on enzyme activity was determined at 30°C using 50 mM of the following buffers: citrate; acetate; MES; MOPS; HEPES; Tris-HCl; CHES; CAPS. The highest activity observed at pH 7.7 is defined as the 100% activity to calculate the relative activity. The temperature profile of the purified enzyme was determined from a range of 0-100°C in a standard assay activity check. In every case, activity was expressed as a percentage of the activity obtained at either the optimum pH or the optimum temperature.

Results and Discussion

Effects of pH and Temperature

The effects of pH on aminopeptidase activity are shown in figures 1 and 2. The optimum pH activity was 8.0 (Fig 3). The enzyme appeared to be stable over a wide pH range of 6.0 to 10.0. The pH optimum and pH stability values were consistent with those obtained by Izawa *et al.* (1997). The optimum temperature activity was 65°C (figure 3) but the enzyme quickly lost activity above 65°C while temperature stability is 40°C (figure 4). The turnover number of this aminopeptidase increases up to 65°C above when the enzyme is no longer stable leading to an irreversible drop in enzyme activity. The enzyme is stable below 40°C for 30 min. This enzyme is therefore considered mesophilic. However, aminopeptidases from mostly of Family III, for example *Bacillus stearothermophilus* have been reported to show good thermostability of up to 90°C, and elevated optimum temperature up to 55°C. (Roncari *et al.*, 1976).

The optimal temperature recorded in this study is close to values for the aminopeptidases reported by Nishiwaki and Hayashi (2001), though these authors reported temperature stability of 55°C. Bertin *et al.* (2005) reported same optimum temperature from *Borrelia burgdorferi*. Optimum pH and pH stability values were in the range as reported by these authors. Contrarily, Sanz and Toldrá (2002) reported optimal activity at pH 5.0 and 37°C for aminopeptidase from *Lactobacillus sakei*. This aminopeptidase, a polar basic amino acid, exclusively hydrolyzes basic amino acids from the amino (N) termini of peptide substrates whereas the aminopeptidase in this study was specific to leucine a non polar hydrophobic amino acid. An aminopeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* was reported to be stable optimally at 100°C (Story *et al.*, 2005).

Conclusion

Enzymes are stable over a limited range of pH and outside this range changes though occur in the charges on ionizable amino acid residues leading to modifications of the tertiary structure of the enzyme. Aminopeptidase from *Aeromonas caviae* is mesophilic with respect to its thermal behaviour hence may not withstand the high temperature during cheese making, and for this reason it is important to seek ways for increasing its thermal stability.

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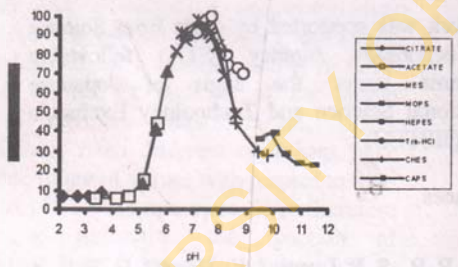

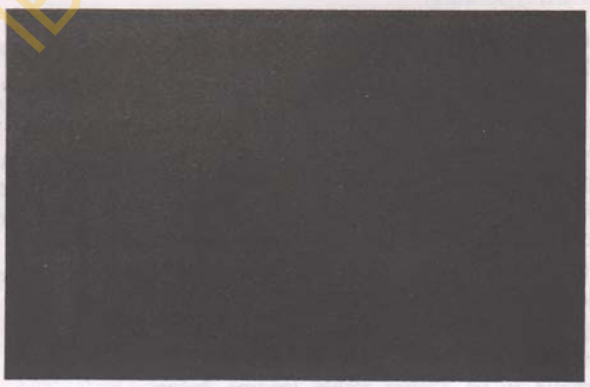

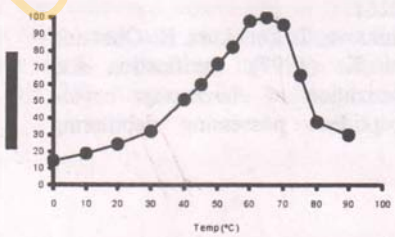

Fig 3. Optimal temperature

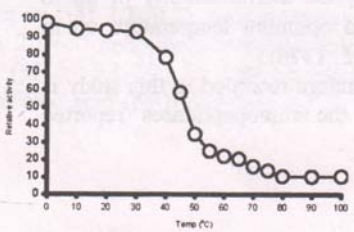

Fig 4. Temperature stability