Properties of aminopeptidase produced by *Euglena pisciformis* cultured with potato protein

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Abstract

Properties of extracellular aminopeptidase from *Euglena pisciformis* cultured with potato protein was studied. Aminopeptidases in the cell membrane and cultured medium exhibited optimum activities at around pH 8, but varied markedly in molecular weight (cytoplasm : 77,000 ; medium, 14,000). The aminopeptidase in cultured medium exhibited optimum activity at around 35℃ and was inactivated at 55℃. *p*CMB potently inhibited the enzyme activity, and 2 mercaptoethanol thoroughly prevented the inhibition. The enzyme was also inactivated by iodoacetamide, and the inactivation was enhanced by the presence of urea.

These results suggest that the extracellular aminopeptidase from *E. pisciformis* contains free sulfhydryl group(s) which is required for the enzyme activity and is buried inside the folded protein.

Key words : alkylating agent, aminopeptidase, degradation, *Euglena pisciformis*, potato, protein, sulfhydryl reagent.

Introduction

Disposal of potato juice is a burden for starch manufacture. NAKAYAMA[3] reported that *Euglena* was effective for disposing the waste water. In recent years, *Euglena pisciformis* was found to proliferate in cesspools in starch factories. We reported that *E. pisciformis* utilizes potato-protein as the sole source of nitrogen after degradation by extracellular aminopeptidase[4, 5]. The present study deals with the properties of the aminopeptidase from *E. pisciformis*.

Materials and Methods

Organism used

E. pisciformis isolated from a cesspool at the Tokachi Tôbu Starch Factory in Urahoro, Hokkaido, was used.

Culture of E. pisciformis

E. pisciformis was cultured in 0.5 L of a medium shown in Table 1 for 14 days. The medium was agitated using a magnetic stirrer at 20℃ in 3,000 lux. Contamination of other microorganisms into the culture medium was checked by culturing the medium on nutrient agar plates.

Preparation of potato protein

Potato tubers (1 kg) were sliced and homogenized in 1.2 L of 10mM sodium ascorbate using a mixer. After squeezing through three layers of gauze, the homogenate was filtrated through

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a filter paper under reduced pressure. Solid ammonium sulfate was added to the supernatant up to 80% saturation, followed by standing at 5℃ overnight. The precipitate was collected by centrifugation at 8,000 *g* for 15 min and dialyzed against deionized water for 3 days. After centrifugation, the supernatant was used as potato protein solution.

Table 1. Composition of basal medium.

Compound	Concentration (mg/L)
$(KH_4)_2HPO_4$	1,000
KH_2PO_4	1,000
$MgSO_4 \cdot 7H_2O$	409
$CaCl_2 \cdot 2H_2O$	26
$FeSO_4 \cdot 7H_2O$	3.3
$MnCl_2 \cdot 4H_2O$	1.8
$CoCl_2 \cdot 6H_2O$	1.1
$ZnSO_4 \cdot 7H_2O$	0.4
$Na_2MoO_4 \cdot 2H_2O$	0.3
$CuSO_4 \cdot 5H_2O$	0.02
Na_3-citrate $\cdot 2H_2O$	516
Vitamin B_1	0.2
Vitamin B_{12}	0.001
Potato protein	3.0

Preparation of crude enzymes

Crude enzymes were prepared from cytoplasm of *E. pisciformis* and the cultured medium. *E. pisciformis* cultured as mentioned above was collected by centrifugation at 1,500 *g* for 10 sec. After washing with 50 *ml* of distilled water by centrifugation at 1,500 *g* for 10 sec, the precipitate was homogenized in 6 *ml* of Na phosphate buffer (50 mM, pH 7.0) on iced water using an ultrasonic generator, followed by centrifugation at 1,000 *g* for 30 min. After twice centrifugation at 20,000 *g* for 30 min, the supernatant was used as the cytoplasm enzyme.

Human *r*-globulin (150 mg) and ammonium sulfate were added successively to the supernatant of the cultured medium (500 *ml*). The precipitate produced between 20 and 80% saturation of ammonium sulfate was collected by centrifugation at 10,000 *g* for 30 min and dialyzed against the buffer for 1 day. The dialyzate was used as the medium enzyme.

Assay of aminopeptidase activity

The reaction mixture consisted of 2 *ml* of Tris-HCl buffer (50 mM, pH 8.0) containing 2 mM leucine-nitroanilide and 0.2 *ml* of crude enzyme. The mixture was incubated at 30℃ for 5 h, followed by adding 1*ml* of 30% acetic acid. *P*-Nitroaniline released was measured at 410 nm.

Gel chromatography

Enzyme solution was loaded onto a Sephadex G-100 column (1.5 x 58 cm) equilibrated with Na-phosphate buffer (50 mM, pH 7.0) containing 0.1 M NaCl and eluted with the buffer at a flow rate of 7.5 *ml*/h. The eluate was collected in 2-*ml* fractions.

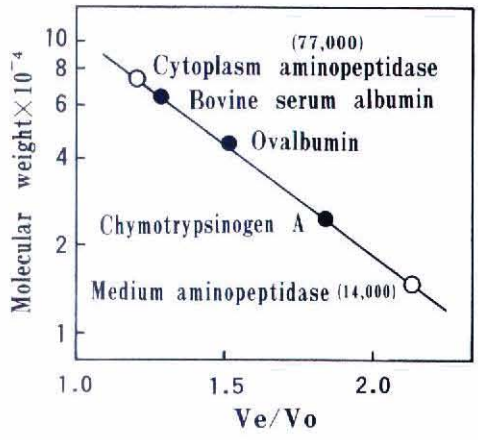

Fig. 1 Determination of the molecular weight of aminopeptidases from cytoplasm of *E. pisciformis* and cultured medium by column chromatography on Sephadex G-100. Vo, void volum ; Ve, elution volume.

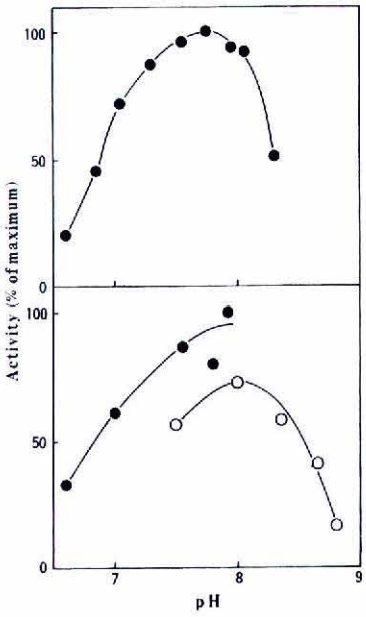

Fig. 2 Effects of pH on activities of aminopeptidases from cytoplasm of *E. pisciformis* and cultured medium. (●) *and* (○) represent activities using McIlvaine and 50 mM Veronal buffers, respectively.

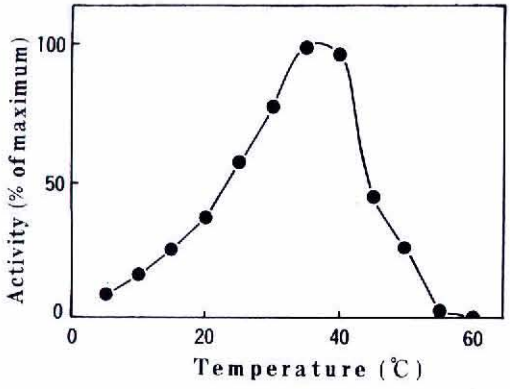

Fig. 3 Effects of temperature on activity of aminopeptidase from cultured medium.

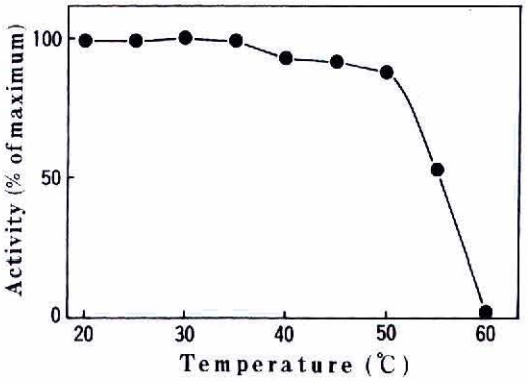

Fig. 4 Thermostability of aminopeptidase from cultured medium. Enzyme solution (0.2 mℓ) was treated at a given temperature for 20 min.

Results

Molecular weight of aminopeptidase

Molecular weights of the aminopeptidases in the cytoplasm and supernatant were 77,000 and 14,000, respectively, as determined by gel chromatography (Fig. 1).

Effect of pH on aminopeptidase activities

Aminopeptidases in the cytoplasm and medium exhibited optimum activities at pH 7.5 and 8.0, respectively (Fig. 2).

Effect of temperature on aminopeptidase activity

The medium aminopeptidase exhibited an optimum activity at 35℃ (Fig. 3). The enzyme activity decreased drastically at 55℃ and was completely lost at 60℃ in the absence of substrate (Fig. 4).

Effect of pCMB and 2-mercaptoethanol on aminopeptidase activity

The medium aminopeptidase activity was potently inhibited by 625 μM pCMB (Table 2). The inhibition of the enzyme was thoroughly prevented by the presence of 25mM 2-mercaptoethanol.

Effect of metal ions and EDTA on aminopep-

tidase activity

The effects of the metal ions on the medium aminopeptidase activity were examined at 2 mM (Table 3). Among the ions tested, Zn^{2+} and Fe^{2+} inhibited 52 and 35% of the activity, respectively. A slight inhibition was also observed by treatment with Mo^{2+}, Cu^{2+} or Ca^{2+}. On the other hand, Co^{2+} slightly enhanced the activity, while EDTA slightly inhibited the activity.

Effect of iodoacetamide and urea on aminopeptidase activity

Alkylation of the medium aminopeptidase by 0.1 M iodoacetamide inhibited 51% of the activity (Table 4). When the alkylation was performed in the presence of 1M urea, 94% of the activity was inhibited.

Table 2. Effects of ρCMB and 2-mercaptoehanol on activity of medium aminopeptidase.

Additive	Activity (% of control)
ρCMB	2
2-Mercaptoehanol	109
ρCMB+2-Mercaptoethanol	104

The enzyme was incubated in 625 μM ρCMB and 25mM 2-mercaptoethanol at 20℃ for 2 h.

Table 3. Effects of metal ions and EDTA on activity of medium aminopeptidase.

Additive	Activity (% of control)
Mn^{2+}(as chloride)	93
Zn^{2+}(as sulfate)	48
Fe^{2+}(as sulfate)	65
Mg^{2+}(as sulfate)	99
Ca^{2+}(as chloride)	85
Cu^{2+}(as sulfate)	78
Co^{2+}(as chloride)	120
Mo^{6+}(as sodium oxide)	73
EDTA (2Na)	89

Final concentrations of the additives were 2 mM.

Table 4. Effects of iodoacetamide and urea on activity of medium aminopeptidase.

Additive	Activity (% of control)
Iodoacetamide	49
Urea	76
Iodoacetamide+Urea	6

The enzyme was incubated in 0.1 M iodoacetamide and 1 M urea at 20℃ for 12 h.

Discussion

In the previous paper, we suggested that *Euglena pisciformis* absorbs potato protein after degradation by extracellular aminopeptidase[4, 5]. The present study dealt with the properties of the aminopeptidase. Aminopeptidases in the cytoplasm and medium exhibited optimum activities at around pH 8 (Fig. 2), while varied markedly in molecular weight (Fig. 1).

The medium aminopeptidase exhibited optimum activity at 35℃, and was inactivated at 55 ℃ (Figs 3 and 4). Therefore, the enzyme seems to be stable at its temperature optimum. In cold regions as Hokkaido, maintenance of the temperature in cesspools will be significant for the enzyme to function.

The aminopeptidase activity was potently inhibited by low concentration of ρCMB, and relatively slightly by 2 mM metal ions (Tables 2 and 3). The inhibition by ρCMB was completely recovered by large excess of 2-mercaptoethanol (Table 2). These observations imply that ρCMB interacts with cysteine required for the activity. The recovering of the activity by large excess of 2-mercaptoethanol may involve the displacement of ρCMB from cysteine residues by 2-mercaptoethanol. The aminopeptidase activity in enzyme preparation was also enhanced by 2-mercaptoethanol (Table 2). This enhancement suggests that some enzymes had been catalytically unfunctional by oxidation of the sulfhydryl groups and 2-mercaptoethanol acti-

vated the unfunctional enzymes by reducing the oxidized groups.

The aminopeptidase was also inactivated by treatment with iodoacetamide, confirming that a free sulfhydryl group is required for the activity (Table 4). The inactivation was enhanced dramatically in the presence of urea (Table 4). The enhancement by the denaturant suggests that sulfhydryl group involved in enzyme activity is buried inside the folded protein[1].

The present study indicated that *Euglena pisciformis* mainly secretes extracellular aminopeptidase exhibiting weak activity at low temperature. It is generally recognized that the degradation of high polymer is enhanced by concerted action of exo- and endo-hydrolases. NAKANO *et al.*[2] reported that *E. gracilis* Z. secretes endo-protease. With respect to disposal of proteins in potato juice, cooperation of microorganisms producing endo- and exo-proteases will be a subject in the future.

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バレイショ蛋白質との培養により産生される*Euglena pisciformis*菌体外アミノペプチダーゼの特性

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摘　　要

バレイショ蛋白質との培養により産生される，*Euglena pisciformis*菌体外アミノペプチダーゼの特性を調べた。細胞質および培地に存在するアミノペプチダーゼの至適pHは共に8付近であったが，分子量は大きく異なった。培地のアミノペプチダーゼは35℃で最大活性を示し，55℃で失活した。pCMBは本酵素を阻害し，2－メルカプトエタノールは阻害を完全に抑えた。ヨードアセトアミドは酵素を失活させ，尿素はこれを助長した。

以上の結果から，*E. pisciformis*の菌体外アミノペプチダーゼは，活性発現にSH基を必要とし，この基は酵素蛋白質の中に折り込まれていると考えられる。

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