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Note

Purification and Some Properties of an Aminopeptidase from the Seeds of *Cannabis sativa*

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An aminopeptidase (HSA) with a molecular mass of 78 kDa was purified from hemp (*Cannabis sativa*) seeds. The activity was inhibited by monoiodeacetic acid, *p*-chloromercuri-phenylsulfonic acid, and Zn^{2+} ion. The specificity of HSA was similar to that of a leucyl aminopeptidase [EC 3.4.11.1] from mammalian cytosol. However, other enzyme properties were different from these of leucyl aminopeptidase.

Key words: *Cannabis sativa*; hemp seed; aminopeptidase; substrate specificity

Aminopeptidases from plants have been isolated and characterized from various sources.[1] Most reports describe aminopeptidases from seeds and leaves. Aminopeptidases from seeds are similar to each other in their properties, especially their substrate specificity, which is comparable to membrane alanyl aminopeptidase [EC 3.4.11.2] from mammals.[1] On the other hand, characterization of leucyl aminopeptidase [EC 3.4.11.1] from a plant source was rare.[2–6] The cDNA sequence of a leucine aminopeptidase, which is an inducible component of the defense response in *Lycopersicon esculentum*, has been analyzed,[7] and the enzymatic characteristics of this aminopeptidase have been studied.[8]

During screening for aminopeptidases from plant sources by using Leu-*p*NA as a substrate, we found a new aminopeptidase from hemp seeds. This paper describes the purification and some characterization of this aminopeptidase (HSA).

Hemp seeds (*Cannabis sativa*) were obtained from Kagoshima City. Hemp seeds (2500 g) were homogenized with a domestic mixer in 2500 ml of distilled water. The homogenate was filtered through a cotton cloth and was centrifuged ($4000 \times g$, for 30 min). Ammonium sulfate fractionation of the supernatant was done, and the 30–60% saturation-fraction was dialyzed and put on a DEAE-cellulose column (3.3×27 cm) equilibrated with buffer A (50 mM Na, K-Pi buffer, pH 7.5). The enzyme was chromatographed with a linear gradient from buffer A (1 l) to buffer B (0.2 M Na, K-Pi buffer, pH 7.5, containing 0.3 M NaCl, 1 l). The active fraction was put on a Sephadex G-200 column (3.3×124 cm) equilibrated with buffer A. The active fraction from the column was put on Sepabeads FP-OT_{13} (Mitsubishi Chemicals, Tokyo) column (1.8×36 cm) equilibrated with buffer C (50 mM Na, K-Pi buffer, pH 7.0, containing 30% saturated ammonium sulfate). The enzyme was eluted with a linear gradient from buffer C (500 ml) to buffer D (50 mM Na, K-Pi buffer, pH 7.0, 500 ml). The enzyme fraction was dialyzed against buffer E (20 mM Na, K-Pi buffer, pH 6.0) and put on a column of Leu-Sepharose (1.1×7.0 cm) equilibrated with buffer E. After the column was washed with 30 mM Na, K-Pi buffer, pH 6.0, then the enzyme was eluited with 50 mM Na, K-Pi buffer, pH 6.0, and stored at $-20°C$. From 2500 g of hemp seeds, 2.6 mg of the purified enzyme was obtained with a final yield of 6.8% (Table 1). SDS-PAGE of the purified protein from the Leu-Sepharose migrated as a single band having a molecular mass of 78 kDa (Fig. 1).

The optimum pH of the protease was about 7 with Leu-*p*NA as a substrate.

To measure the pH stability of the enzyme, the incubation mixture consisted of 0.2 ml of the enzyme solution (60 μg/ml) in buffers of various pHs. The assay solution was incubated at 37°C for 60 min. The residual activity of the enzyme was assayed after the various pH solutions were returned to neutral pH with buffer F (10 mM Na, K-Pi buffer, pH 7.0) by the assay method described above using Leu-*p*NA as a

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Abbreviations: DFP, diisopropyl fluorophosphate; HSA, hemp seed aminopeptidase; Na, K-Pi buffer, sodium-potassium phosphate buffer; PCMPS, *p*-chloromercuriphenylsulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; *p*NA, *p*-nitroanilide

Table 1. Purification of Hemp Seed Aminopeptidase

Purification step	Total protein (mg)	Total activity ($\times 10^{-6}$ units)	Specific activity ($\times 10^{-3}$ units/mg)	Recovery (%)	Purity (fold)
Ammonium sulfate ppt.	17,800	6.78	0.380	100	1
DEAE-cellulose	211	3.43	16.3	51	43
Sephadex G-200	115	2.24	19.5	33	51
Sepabeads FP-OT_{13}	22.3	2.02	90.6	30	238
1st Leu-Sepharose	3.40	0.570	168	8	442
2nd Leu-Sepharose	2.56	0.462	180	7	474

Aminopeptidase was purified from 2500 g of hemp seeds.

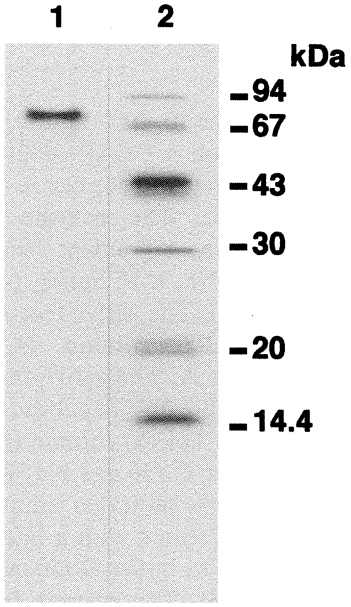

Fig. 1. SDS-PAGE of Purified HSA.

SDS-PAGE was done by the method of Laemmli.[9] The samples were electrophoresed in 15% polyacrylamide gel. After electrophoresis, the protein was stained with 0.1% Coomassie Brilliant Blue R-250 for 15 min and then destained. Lane 1, molecular mass markers. The molecular mass markers used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactoalbumin (14 kDa). Lane 2, 10 μg of pooled fractions from the rechromatography on a Leu-Sepharose column.

substrate. At least 80% of the activity remained after incubation between pH 6 and 8.

To examine the effects of temperature on the stability of the enzyme, the residual activities of the enzyme were measured after the enzyme (0.5 ml) was incubated in buffer B at various temperatures (20–70°C) for 60 min. The assay was done by the assay method for protease described above with Leu-*p*NA as a substrate. The enzyme was fairly stable up to 50°C.

The effects of various inhibitors on the enzyme activity toward Leu-*p*NA as a substrate were tested. The enzyme (0.2 ml, 60 μg/ml) was incubated with 0.2 ml of 0.2 M Tris-HCl buffer, pH 7.5, containing

Table 2. Hydrolysis of Amino Acid-*p*NA with Hemp Seed Aminopeptidase

Substrates	Hydrolysis rate ($\times 10^{-3}$ units/min/mg protein)	Relative activity (%)
Leu-*p*NA	180	100[a]
Ala-*p*NA	74.7	42
Pro-*p*NA	54.7	30
Gly-*p*NA	3.42	2
Arg-*p*NA	1.62	1
Lys-*p*NA	1.26	1
Glu-*p*NA	0	0

[a] The enzyme activity toward Leu-*p*NA is shown as 100%.

various inhibitors at 37°C. After 120 min, the remaining activity was assayed by the method mentioned above with Leu-*p*NA as a substrate. The control assay was done with an enzyme solution without inhibitor. HSA activity was inhibited until only 30% of the original activity remained, by incubation with 0.2 mM PCMPS or 0.2 mM $ZnSO_4$ at 37°C for 60 min, and completely inactivated by incubation with these compounds for 120 min. The activity was slightly inhibited by monoiodoacetic acid (2.0 mM), $MnSO_4$ (2.0 mM), and $CoSO_4$ (2.0 mM). Under similar conditions, 1, 10-phenanthroline, EDTA, EGTA, PMSF, and DFP had no effect on the activity. Bestatin (0.02 mM) caused no inhibition. The results indicated that the protease may be a cysteine type. Cathepsin H, acts as both an aminopeptidase and an endopeptidase with a molecular mass of 28 kDa, is a cysteine protease of the papain family.[10,11] N-blocked substrates were not hydrolyzed by HSA (data not shown), and the molecular mass of HSA (78 kDa) is larger than that of cathepsin H. Aminopeptidase C (PepC) of lactic acid bacteria is also classified as a cysteine peptidase.[12]

The proteolytic activity using Leu-*p*NA as a substrate was measured by the method previously reported by Yonezawa *et al.*[13] The hydrolysis rates and kinetic parameters of hydrolysis of several amino-acid-*p*NA substrates by HSA were measured (see Table 2). The best substrate for the protease was Leu-*p*NA. Ala-*p*NA and Pro-*p*NA were substrates for HSA. Gly-*p*NA, Arg-*p*NA, and Lys-*p*NA were

slightly hydrolyzed by the enzyme. Glu-*p*NA was not hydrolyzed by HSA. The kinetic parameters (K_m) of the hydrolysis of (Leu, Pro, and Ala)-*p*NA by HSA were 0.248, 0.194, and 8.78 (μM), respectively. A synthetic oligopeptide, Leu-Gly-Tyr-Leu (100 μl, 400 nmol), was hydrolyzed by HSA (100 μl, 0.2 nmol) in 0.1 ml of 20 mM Na, K-Pi buffer, pH 7.0, at 37°C. At intervals for 30 min, portions (10 μl) were taken out and the reaction was stopped in them by the addition of 10 μl of 0.1 M HCl. The hydrolysates were derivatized with phenylisothiocyanate in a Pico·Tag work station (Waters). Phenylthiocarbamyl derivatives of the amino acids were analyzed on a Pico·Tag HPLC amino acid analysis system (Waters). A large amount (70%) of Leu residue of the peptide was measured by hydrolysis at 37°C for 2 h with HSA. The amounts of amino acid released from the tetrapeptide were Leu > Gly > Tyr. It suggested that the amino acids, Leu, Gly, and Tyr were successively released from the N-terminal of the peptide by HSA. These results showed that the specificity of HSA toward amino-acid-*p*NAs as substrates, was similar to that of leucyl aminopeptidase from bovine lens.[14] However, other enzyme properties were different from these of leucyl aminopeptidase. Two aminopeptidases, AP1 (58 kDa) and AP2 (74 kDa) have been identified in peas.[15] These enzyme properties such as effects of inhibitors and pH-activity profiles were similar to these of HSA. The substrate specificity of AP1 was similar to these of HSA or leucyl aminopeptidase from bovine lens, on the other hand, AP2 showed a broad specificity. The molecular mass of AP2 is particularly close to that of HSA. Plant aminopeptidases have been classified into two classes, "naphthylamidase type" and "leucine aminopeptidase type" based on enzyme properties by Bartling and Weiler.[16] "Naphthylamidase type" aminopeptidases with molecular masses of 60–90 kDa were insensitive to metal chelators but inhibited by SH-reagents. "Leucine aminopeptidase type" enzymes were inhibited by metal chelators but unaffected by SH-reagents.[17] These properties of "Naphthylamidase type" were similar to these of HSA, but the substrate specificity of "Naphthylamidase type" enzymes except for pea aminopeptidase as stated abobe were not similar to that of HSA. It seems that HSA is unclassifiable into these two classes.

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