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Purification and Characterization of an Aminopeptidase, the Enzyme-Hydrolyzing Alanine-*p*-nitroanilide (APAase), from *Euonymus* Leaves

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An aminopeptidase, the enzyme-hydrolyzing alanine-*p*-nitroanilide (APAase), from leaves of *Euonymus alatus* f. *ciliato-dentatus* was purified about 1,400-fold by a combination of ion-exchange and gel filtration column chromatographies. Polyacrylamide gel electrophoresis showed the purified APAase to be homogenous. The molecular weight of this APAase was estimated to be about 100,000, and the optimum pH for its hydrolytic activity against alanine-*p*-nitroanilide (APA) was 8.6–9.0. APAase hydrolyzed alanine-β-naphthylamide (alanine-NA), glycine-NA, lysine-NA and arginine-NA. It was inhibited slightly by *p*-chloromercuribenzoate (PCMB) and iodoacetic acid and was not activated by thiol reagents. Therefore, a sulfhydryl group could not be present at the active site of APAase. APAase was inhibited strongly by 1,10-phenanthroline, but was unaffected by EDTA. Of the metal ions tested, Hg^{2+}, Zn^{2+} and Mn^{2+} strongly inhibited its activity, and Ca^{2+} stimulated it to some extent.

Key words: Aminopeptidase — Enzyme-hydrolyzing alan ine-*p*-nitroanilide (APAase) — *Euonymus alatus* (leaf) — Substrate specificity.

The roles of various aminopeptidases in protein turnover in germinating seeds and senescent leaves have been studied (Sopanen and Lauriere 1976, Tully and Beeveres 1978, Leung and Bewley 1983). Some of those enzymes found in various plants show substrate specificity against LPA and leucine-NA (Kolehmainen and Mikola 1971, Elleman 1974, Tomomatsu et al. 1978, du Toit and Schabort 1978, Ikeda et al. 1983). In addition, an aminopeptidase which shows higher activity against APA and alanine-NA than against LPA and leucine-NA has been studied in human tissues that has been referred to as alanine aminopeptidase (Behal et al. 1966, Starnes and Behal 1974, Starnes et al. 1982).

Recently, similar enzymes have been found in several plants (Vodkin and Scandalios 1980, Malik 1983, Waters and Dalling 1984), but detailed studies of those enzymes have yet to be made. In a previous paper (Tazaki and Ishikura 1983), we reported that *Euonymus* leaves contain three different aminopeptidases; LPAase 1, LPAase 2 and APAase, and that both LPAase 1 and 2 hydrolyze LPA, whereas APAase hydrolyzes APA. Of those enzymes, LPAase 2, which showed the highest activity in *Euonymus* leaves, was purified and its properties examined (Tazaki and Ishikura 1984). We now describe the purification and characterization of the APAase in *Euonymus* leaves.

Abbreviations: APA, alanine-*p*-nitroanilide; APAase, enzyme-hydrolyzing APA; KPB, potassium phosphate buffer; LPA, leucine-*p*-nitroanilide; LPAase, enzyme-hydrolyzing LPA; NA, β-naphthylamide; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

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Materials and Methods

Plant materials—Leaves of *Euonymus alatus* (Thunb.) Sieb. f. *ciliato-dentatus* (Fr. et Sav.) Hiyama were collected from a single tree in mid-November 1983.

Enzyme extraction—Extraction of the enzyme from *Euonymus* leaves (300 g) and Sephadex G-25 column chromatography to remove the low-molecular materials in the extract were performed as described elsewhere (Tazaki and Ishikura 1983). The enzyme solution eluted was used as the crude extract. All purification procedures were done at 0–2°C.

Enzyme purification—The crude extract was applied to a DEAE-cellulose column (32× 100 mm) that had been equilibrated with 0.01 M KPB (pH 7.0), then APAase was eluted with 0.4 M KCl in the same buffer. Ammonium sulfate was added to the eluted enzyme solution, and the fraction between 40–80% was collected as a precipitate by centrifugation then dissolved in a small volume of 0.01 M KPB (pH 7.0), followed by desalting on a Sephadex G-25 column. The desalted enzyme solution was applied to a second DEAE-cellulose column (22×160 mm) and eluted with a linear gradient of 0 to 0.3 M KCl in 0.01 M KPB (pH 7.0). The pooled eluates containing APAase was brought to 80% saturation with ammonium sulfate then centrifuged. The protein precipitates were dissolved in a small volume of 0.01 M KPB (pH 7.0) containing 0.2 M NaCl.

This enzyme solution was applied to a Sephadex G-150 column (27×960 mm) that had been equilibrated with 0.01 M KPB (pH 7.0) containing 0.2 M NaCl. The crude enzyme (fractions No. 90–100) was eluted with the same buffer then desalting on a Sephadex G-25 column.

The desalted solution was layered on a DEAE-Sepharose column (22×160 mm) that had been equilibrated with 0.01 M KPB (pH 7.0), after which the enzyme was eluted with a linear gradient of 0 to 0.3 M KCl in the same buffer. The enzyme (fractions No. 95–101) was collected and concentrated to a small volume with a Mini-module NM-3 hollow fiber membrane.

This concentrate was applied to a Sephacryl S-300 column (19×900 mm) that had been equilibrated with 0.02 M KPB (pH 7.6) containing 0.5 M NaCl and 10% glycerol, then the enzyme was eluted with the same buffer. Fractions (No. 60–63) that contained the enzyme were combined and stored in 50% (v/v) glycerol at −20°C.

Enzyme assay—Unless otherwise stated, the APAase and LPAase activities were assayed as described earlier (Tazaki and Ishikura 1983). One unit of activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per min at pH 8.0 and 30°C.

Determination of protein—The enzyme was analyzed by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Determination of molecular weight—The molecular weight of the APAase was estimated by gel filtration on a Sephadex G-150 column (27×960 mm). Ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), albumin (67,000) and aldolase (158,000) were used as the standards of molecular weight.

Polyacrylamide gel electrophoresis—Disc polyacrylamide gel electrophoresis and staining to detect APAase activity were done as described previously (Tazaki and Ishikura 1983), except that alanine-NA was dissolved in 0.05 M Tris-HCl buffer (pH 8.6). The enzyme was stained with Coomassie brilliant blue R-250.

Chemicals—APA was purchased from the Protein Research Foundation, Osaka; LPA and leucine-NA from Wako Pure Chemical Ind., Tokyo; phenylalanine-NA from Aldrich Chemical Co., Wisconsin, U.S.A.; and the other aminoacyl-NAs from Sigma Chemical Co., Missouri, U.S.A.

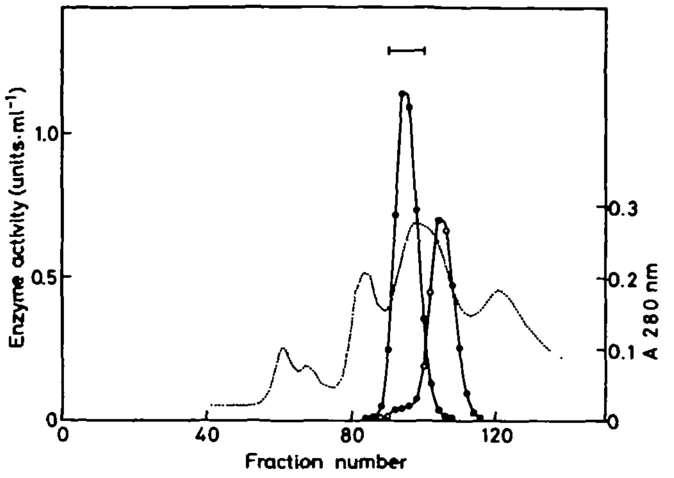

Fig. 1 Chromatography of APAase on a Sephadex G-150 column. The enzyme fraction obtained by the second DEAE-cellulose column chromatography was eluted through a Sephadex G-150 column with 0.01 M KPB (pH 7.0) containing 0.2 M NaCl. Each fraction was 3 ml. Enzyme activities with APA (●) and LPA (○); A_{280} (----).

Results

Purification of APAase—As reported elsewhere (Tazaki and Ishikura 1984), APAase was not separated completely from LPAase 1 and LPAase 2 by the second DEAE-cellulose column chromatography. Further chromatography on a Sephadex G-150 column showed that APAase was eluted as a single active peak, whereas its activity against LPA gave two peaks, a major and minor one (Fig. 1). The minor peak coincided with the APAase activity peak; therefore, APAase also may have the activity against LPA although with a very low rate. This was verified by chromatography on a DEAE-Sepharose column, which gave an elution profile with

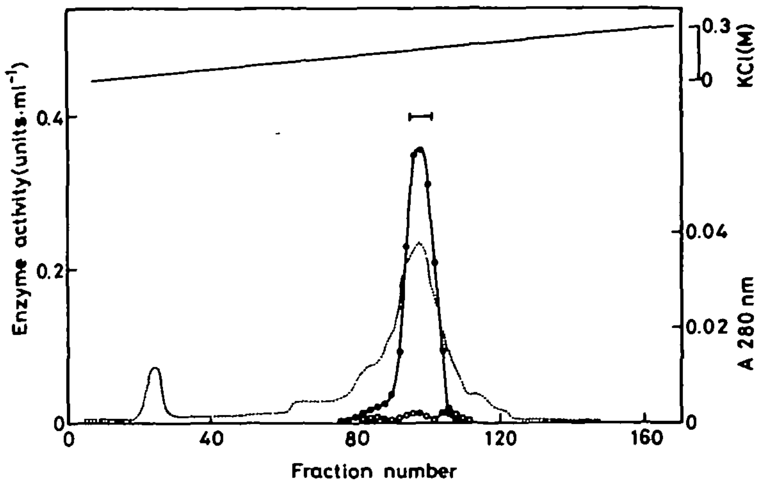

Fig. 2 Chromatography of APAase on a DEAE-Sepharose column. The enzyme fractions obtained by Sephadex G-150 column chromatography were run through a DEAE-Sepharose column using a linear gradient of 0 to 0.3 M KCl in 0.01 M KPB (pH 7.0). Each fraction was 6 ml. Enzyme activities with APA (●) and LPA (○); A_{280} (----); KCl concentration (——).

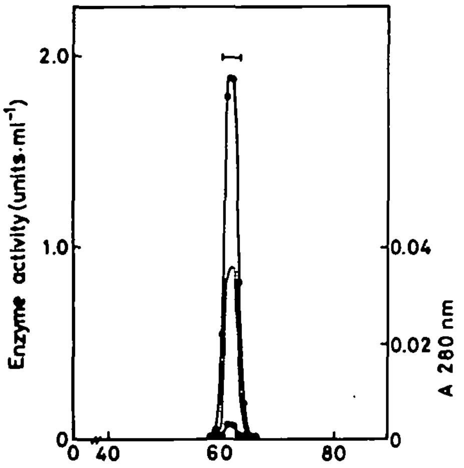

Fig. 3

Fig. 4

Fig. 3 Chromatography of APAase on a Sephacryl S-300 column. The enzyme fraction obtained by DEAE-Sepharose column chromatography was run through a Sephacryl S-300 column, the enzyme being eluted with 0.02 M KPB (pH 7.6) containing 0.5 M NaCl and 10% glycerol. Each fraction was 2.5 ml. Enzyme activities with APA (●) and LPA (○); A_{280} (----).

Fig. 4 Polyacrylamide gel electrophoresis of purified *Euonymus* APAase. The enzyme (7 μg) was applied to the gel for protein staining. Staining for APAase activity (A) was done with alanine-NA, and for protein (B) with Coomasie brilliant blue R-250.

three active peaks against LPA that had very low activity (Fig. 2). The middle peak coincided with that of APAase activity against LPA. APAase was purified as the final step of chromatography on a Sephacryl S-300 column, and the purified enzyme still showed low activity against LPA (Fig. 3). The APAase activity against LPA was 10% of that against APA when its activity was assayed at pH 8.6.

Purification procedures used with the enzyme are shown in Table 1. The APAase from the crude extract was purified about 1,400-fold, yielding 12% of the activity. On disc polyacrylamide gel electrophoresis, the purified enzyme gave only one protein band which coincided with that for APAase activity (Fig. 4). Activity was stable for at least three months when the purified enzyme was stored in 50% (v/v) glycerol at −20°C.

Characterization of APAase—The effect of pH on APAase activity is shown in Fig. 5. APAase

Table 1 Purification of APAase from *Euonymus* leaves

Purification step	Protein (mg)	Total activity (units)	Specific activity (units·mg-protein^{-1})	Yield (%)	Purification (−fold)
Crude extract	3,039	103	0.0339	100	
1st DEAE-cellulose	501	77.3	0.154	75	4.5
40–70% $(NH_4)_2SO_4$	337	61.9	0.183	60	5.4
2nd DEAE-cellulose	41.4	31.7	0.766	30.7	22.6
Sephadex G-150	4.40	24.8	5.64	24.0	166
DEAE-Sepharose	1.13	13.6	12.1	13.2	356
Sephacryl S-300	0.260	12.5	48.1	12.1	1,417

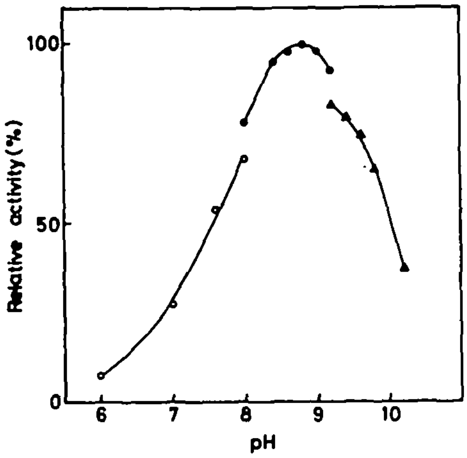

Fig. 5

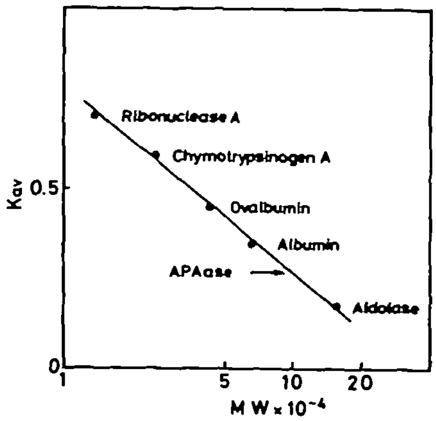

Fig. 6

Fig. 5 Effect of pH on APAase activity. The enzyme activity against APA was assayed in 0.1 M KPB (○), 0.1 M Tris-HCl buffer (●) and 0.1 M glycine-NaOH buffer (▲).

Fig. 6 Estimation of the molecular weight of APAase on the Sephadex G-150 column. Kav values for each protein were calculated from the equation, Kav=Ve−Vo/Vt−Vo, in which Ve is the elution volume for the protein, Vo the column void volume and Vt the total bed volume.

showed a broad pH optimum at 8.6–9.0. Therefore, we assayed APAase activity at pH 8.6 in the following experiments.

The molecular weight of APAase was estimated as about 100,000 by gel chromatography on a Sephadex G-150 column (Fig. 6).

Effects of various metal ions on the enzyme's activity are presented in Table 2. APAase was inhibited strongly by Mn^{2+}, Zn^{2+} and Hg^{2+}. None of the metal ions tested stimulated APAase, but Ca^{2+} enhanced its activity to some extent.

The effects of various reagents on the APAase activity are given in Table 3. Of the reagents tested, the most inhibitory was 1,10-phenanthroline. APAase was inhibited slightly by PCMB, TPCK and PMSF, and by iodoacetic acid at a high concentration, but there was little affection with the other reagents.

Table 2 Effects of various metal ions on APAase activity

Reagent	Relative activity (%)
None	100
$MgCl_2$	100
$CaCl_2$	122
$BaCl_2$	91
$CoCl_2$	38
$CuCl_2$	35
$MnCl_2$	12
$ZnCl_2$	0
$HgCl_2$	0

Enzyme activities against APA were assayed in the presence of 1 mM metal ion. Activities are given as percents of the control.

Table 3 Effects of various inhibitors and activators on APAase activity

Reagent	Concentration (mM)	Relative activity (%)
None		100
PCMB	0.1	71
Iodoacetic acid	1	112
	5	68
N-Ethylmaleimide	1	103
	5	97
Dithiothreitol	1	112
	5	95
β-Mercaptoethanol	1	119
	5	111
EDTA	1	110
	5	99
1,10-Phenanthroline	1	53
	5	18
TPCK	0.5	72
PMSF	0.5	70

After the enzyme had been incubated for 30 min at 0°C with inhibitors or activators at the indicated concentration. APAase activity was assayed with APA. Activities are given as a percent of the control.

Table 4 Hydrolysis of various substrates by APAase and LPAase 2

Substrate	Relative activity (%)	
	APAase	LPAase 2
Ala-NA	100	8
Gly-NA	31	0
Lys-NA	30	0
Arg-NA	18	0
Leu-NA	6	100
Phe-NA	5	89
Ser-NA	3	0
Tyr-NA	2	80
Ile-NA	0	5
His-NA	0	0
Pro-NA	0	0
Val-NA	0	0
Asp-NA	0	0
Glu-NA	0	0

The concentration for each substrate was 0.2 mM. The 100% activities of APAase and LPAase respectively are given as the value for Ala-NA hydrolysis and for Leu-NA hydrolysis. APAase activity against APA was 138% of that against Ala-NA.

The reaction rates of APAase with various aminoacyl-NAs are shown in Table 4. To compare the substrate specificity of APAase with that of LPAase 2, we made a parallel examination of the activity of LPAase 2 (prepared as described elsewhere [Tazaki and Ishikura 1984]). The 14 substrates tested fell into three groups. The first group, which included alanine-NA, glycine-NA, lysine-NA and arginine-NA, was defined as containing good substrates for APAase. Leucine-NA, phenylalanine-NA and tyrosine-NA fell in the second group, in which group substrates are efficiently hydrolyzed by LPAase 2. The remaining substrates that made up the third group were only slightly hydrolyzed or not hydrolyzed by the enzymes.

Discussion

The APAase from *Euonymus* leaves was purified about 1,400-fold by a combination of ion-exchange and gel filtration column chromatographies. The purified enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis. Although aminopeptidases similar to APAase recently have been separated and characterized from pea (Elleman 1974), maize (Vodkin and Scandalios 1980) and other plants (cf. Starnes and Behal 1974), the purifications made were insufficient to determine the exact properties of these enzymes.

APAase had a broad pH optimum between 8.6 and 9.0 and a molecular weight of about 100,000. These properties indicate that APAase differs markedly from *Euonymus* LPAase 2, as well as differing from the aminopeptidases of barley (Kolehmainen and Mikola 1971), Japanese apricot (Ninomiya et al. 1981) and wheat (Waters and Dalling 1984). It is, however, very similar to the maize aminopeptidase, AMP 2, with a pH optimum at 8.5 and a molecular weight of about 88,000 (Vodkin and Scandalios 1980).

Euonymus APAase was inhibited slightly by PCMB and iodoacetic acid, but was not affected by thiol reagents; therefore, no sulfhydryl group takes part in the active site of this APAase. APAase activity was inhibited strongly by 1,10-phenanthroline, but the enzyme was not affected by EDTA. Furthermore, none of the metal ions tested stimulated APAase although Ca^{2+} somewhat enhanced its activity. Further study is required to determine whether APAase is a metalloenzyme.

The APAase also was notably different from LPAase 2 in its substrate specificity, being most active against alanine-NA among the 14 substrates tested. It also showed higher activity against glycine-NA, lysine-NA and arginine-NA than against the other substrates. Similar substrate specificity has been shown by AP 2, one of two aminopeptidases from pea (Elleman 1974), and by AMP 2 from maize (Vodkin and Scandalios 1980). *Euonymus* APAase closely resembles the aminopeptidase AMP 2 from maize in several respects; molecular weight, pH optimum and substrate specificity. Vodkin and Scandalios (1980) also showed that dipeptidyl-NA is hydrolyzed more efficiently by a combination of two aminopeptidases than by those enzymes singly, but the substrate specificities differed. Complementary action of different aminopeptidases on natural peptide substrates, therefore, seems to occur in vivo. Because the activities of APAase and LPAase increase concurrently in senescing leaves (Tazaki and Ishikura 1983), this complementary action of the enzymes on peptide substrates may also take place in *Euonymus* leaves.

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