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Purification of an Aminopeptidase Preferentially Releasing N-terminal Alanine from Cucumber Leaves and Its Identification as a Plant Aminopeptidase N

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Note

Purification of an Aminopeptidase Preferentially Releasing N-terminal Alanine from Cucumber Leaves and Its Identification as a Plant Aminopeptidase N

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In this study, a highly active foliar aminopeptidase preferentially releasing N-terminal alanine from artificial substrates was purified and characterized from cucumber (*Cucumis sativus* L. suyo). The enzyme had a molecular mass of 200 kDa consisting of two subunits of 95 kDa. It was a metalloprotease the pH optimum of which was 8 to 9. It cleaved Ala-, Gly-, Met-, Ser-, Leu-, Lys-, and Arg artificial substrates. An internal amino acid sequence was similar to those of aminopeptidase N (clan MA, family M1) of microorganisms, and was very similar to that of a putative aminopeptidase N of *Arabidopsis thaliana*. From these results, the highly active aminopeptidase in cucumber leaves was identified to be a plant aminopepitdase N.

Key words: *Cucumis sativus*; cucumber; purification; aminopeptidase N; naphthylamidase

Aminopeptidase (APase) is an enzyme releasing the amino-terminal amino acid residues from proteins and peptides, and is involved in the turnover of proteins. A well-known physiological role of plant APases is the mobilization of protein reserves in germinating seedlings.[1-3] In addition, recent reports proposed that APase might have other roles in biologically fundamental processes such as governing the half-life of proteins,[4] defense response to infection and wounding,[5] protein maturation,[6] and auxin transport.[7]

APases are classified into many groups on the basis of their primary structures.[8] In plants, however, only the Leu-APase group has been well identified so far,[5,9,10] and other groups have been not well identified despite many studies on plant APases. In cucumber leaves, a highly active APase exists and it is unlikely to belong to the Leu-APase group because it preferentially releases alanine from artificial substrates. The aim of this study is to clearly identify the properties of this not well-identified APase, and decide what group the APase belongs to.

APase activity was monitored by measurement of the cleavage of 1 mM L-aminoacyl-*p*-nitroanilide (pNa, Sigma, St. Louis, Mo, USA) or L-aminoacyl-2-naphthylamide (NA, Sigma) in 50 mM Hepes-KOH, pH 8.0. For routine assay, Ala-pNa was used as a substrate. Formation of nitroaniline and naphthylamine were measured as described previously.[11,12]

Purification of APase was done by the following procedure below 4°C. Mature leaves (50 g) of field-grown cucumber (*Cucumis safivus* L. suyo, purchased from Yamato Noen, Nara, Japan) were frozen in liquid N_2 and homogenized with 1% (w/w) polyvinylpolypyrrolidone and 5 volumes of 50 mM K-PB, pH 7.0, containing 2 mM 2-ME and 1 mM EDTA. After centrifugation at 20,000 *g* for 20 min, the supernatant was fractionated by the addition of solid ammonium sulfate. Protein precipitated between 33–45% ammonium sulfate saturation was dissolved with 5 volumes of 200 mM sodium-acetate buffer, pH 5.0, containing 1 mM 2-ME, and insoluble proteins were removed by centrifugation. After the supernatant was dialyzed against 25 mM K-PB, pH 7.0, containing 1 mM 2-ME (buffer A), the dialysate was put on a DEAE-Toyopearl column (4 × 20 cm, Tosoh Corp., Japan) equilibrated with buffer A. A linear gradient of 0–0.3 M NaCl was used to elute the enzymes. After active fractions were combined, a solution of 80% saturated ammonium sulfate in 50 mM K-PB, pH 7.0, containing 1 mM 2-ME and 0.1 mM EDTA was added to reach a final concentration of 20%. Then the enzyme solution was put on a phenyl-Sepharose column (2 × 15 cm, Amersham Pharmacia Biotech, Sweden) equilibrated with 20% ammonium sulfate saturated in 50 mM K-PB, pH 7.0, containing 1 mM 2-ME and 0.1 mM EDTA and eluted with a linear gradient of 20–0% ammonium sulfate. Active fractions were combined and dialyzed against 10 mM sodium-phosphate buffer (Na-PB), pH 6.8, containing 1 mM 2-ME. The dialysate was put on a hydroxyapatite column (1.5 × 8 cm, Bio-Rad, CA, USA) and eluted by a linear gradient of 0–0.2 M Na-PB, pH 6.8. Active fractions were combined and dialyzed against buffer A, and then put on a Mono Q column (1.5 × 5 cm, Amersham Pharmacia Biotech). After

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Abbreviations: APase, aminopeptidase; 2-ME, 2-mercaptoethanol; NA, 2-naphthylamide; PB, phosphate buffer; pNa, *p*-nitroanilide

elution by a linear gradient of 0–0.2 M NaCl, the active fraction was concentrated by Vivapore-10 (Vivascience Ltd., UK) and put on a gel filtration column (1.5 × 60 cm, HW-55F, Tosoh) equilibrated with buffer A. The active fraction was concentrated by Vivapore-10, then the solution was put through preparative native-PAGE. After electrophoresis, portions containing APase were identified by activity staining for APase and excised by a feather blade. APase in the gel was electroeluted by Centrilutor (Millipore, MA, USA) and concentrated by Microcon-30 (Millipore).

Native-PAGE was done with 3–10% gradient polyacrylamide gels at pH 8.3 by the method of Davis.[13] SDS-PAGE was done by the method of Laemmli using 10% (w/v) acrylamide gel.[14] Protein concentrations were measured by the method of Bradford with bovine serum albumin as the standard.[15] Internal amino acid sequences were analyzed by the method described previously.[12]

Three APase activities were detected by activity staining using three representative artificial substrates for APase (Ala-NA, Met-NA, and Leu-NA) after separation by native-PAGE of cell-free extracts of cucumber leaves (Fig. 1, lane 1–3). Based on their migration, they were named APase1, 2, and 3. APase2 was the most active APase against all substrates. APases1 and 3 were less active than APase2, and they showed narrow substrate specificities, *i.e.* APase1 cleaved Met-NA, and APase3 cleaved Leu-NA. From their substrate specificities, AP1 and AP3 might be categorized into Met-APase and Leu-APase groups, respectively. In the following study, APase2, which we were unable to categorize from its substrate specificity, was purified and characterized.

The major portion of Ala-pNa cleaving activity of crude extract was recovered in the fraction of 33–45% saturated ammonium sulfate precipitation (Table 1). This fraction was purified by successive chromatography on DEAE-Toyopearl, phenyl-Sepharose, hydroxyapatite, Mono Q, and gel filtration columns. In the final step, APase was purified homogeneously by preparative native-PAGE and then electroelution. Migration of the purified APase on native-PAGE was identical to that of APase2 in a crude extract (Fig. 1, lane 4). This APase also showed Met-NA and Leu-NA cleaving activities on activity staining (data not shown). The molecular mass of purified APase2 was estimated to be 95 kDa on SDS-PAGE and 200 kDa by gel filtration (Fig. 2). This suggests that native APase2 is a dimeric protein composed of identical subunits of 95 kDa. Inhibitor analysis suggested that the APase2 was a metalloprotease since *o*-phenanthroline, a metal chelating reagent, completely inhibited the activity, and bestatin, a metalloprotease inhibitor selective for APase, was very effective, but other classes of protease inhibitors did not inhibit it (data not shown). APase2 was

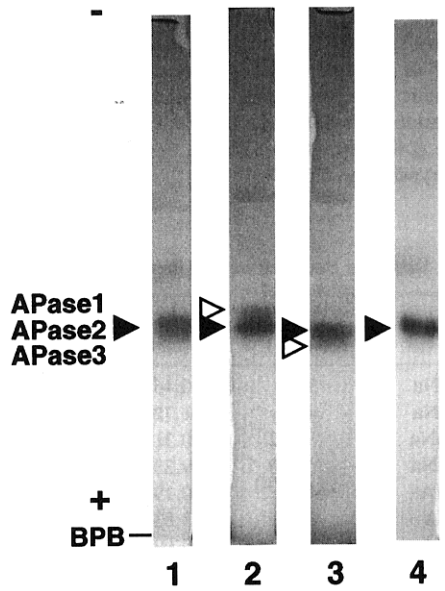

Fig. 1. Activity Staining of APases in Crude Extract and Purified APase2.

After electrophoresis of crude extracts (lane 1–3) and purified APase2 (labe 4) on native-PAGE, gel strips were incubated with 1 mM Ala-NA (lane 1, 4), Met-NA (lane 2) and Leu-NA (lane 3) in 50 mM Hepes-KOH, pH 8.0, at 37°C for 10 min (Ala-NA) or 15 min (Met-NA and Leu-NA). Activities on the gel were stained with 0.1% (w/v) Fast Garnet GBC (Sigma). Closed arrowheads indicate activity bands the migration of which is identical to those of purified APase2. Open arrowheads indicate activity bands derived from APase1 and 3.

Table 1. Purification of APase2

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min·mg)	Yield (%)	Purification (-fold)
Crude extract	842	15800	18.8	100	1
$(NH_4)_2SO_4$ pellet (33–45%)	57.6	9510	165	60.2	8.79
DEAE-Toyopearl	10.5	7220	687	45.7	36.6
Phenyl-Sepharose	3.57	4790	1340	30.1	71.4
Hydroxyapatite	1.05	3970	3780	25.1	201
Mono Q	0.650*	3000	4620	19.0	246
HW-55F	0.168*	1010	6010	6.38	320
Electroelution	0.074*	508	6860	3.22	365

* Protein was estimated by measurement of the absorbance at 280 nm where the $E|^{1\%}_{1cm}$ value is 10.0.

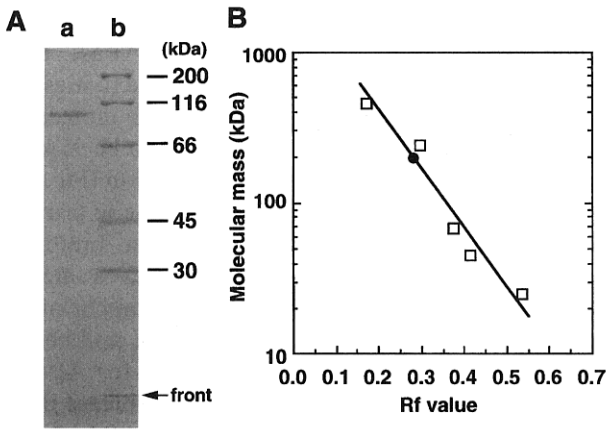

Fig. 2. Molecular Mass of APase2.

(A) Purified APase2 (1 μg, lane a) and protein markers (lane b) were put through SDS-PAGE using 10% acrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250.

(B) Plot of the mobility against molecular mass on a logarithmic scale for APase2 (●) and marker proteins (□) on gel filtration (HW-55F). Marker proteins are ferritin (443 kDa), catalase (240 kDa), BSA (67 kDa), egg albumin (45 kDa), and chymotrypsinogen (25 kDa).

Table 2. Substrate Specificity and Kinetic Parameters of APase2

Substrate	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (sec^{-1}/mM)
Ala-pNa	34.3	0.45	76.2
Gly-pNa	45.2	1.72	26.3
Met-pNa	9.72	0.31	31.4
Leu-pNa	6.70	9.23	0.726
Ala-NA	149	0.197	756
Ser-NA	87.0	7.83	11.1
Lys-NA	23.1	0.092	251
Arg-NA	19.3	0.099	195

Activity was measured in 50 mM Hepes-KOH, pH 8.0 at 37°C. Purified APases showed little or no cleaving activities against Asn-NA, Asp-NA, Cys-pNa, Glu-NA, His-NA, Ile-NA, Phe-NA, Pro-NA, Trp-NA, Tyr-NA, and Val-pNa derivatives.

inhibited by 1 mM Cu^{2+} and Zn^{2+}, but not by Ca^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+} (data not shown). It had a pH optimum at 8 to 9, and a temperature optimum at 37 to 45°C (data not shown). APase2 preferentially released neutral amino acids such as Ala, Gly, Ser, Met, and to lesser extents, Leu, and basic amino acids from L-aminoacyl derivative substrates (Table 2). These biochemical properties resembled those of typical APase N, a metalloprotease that preferentially acts on N-terminal neutral amino acids of peptides.[8,16,17]

Because amino acid sequencing of the N-terminus of APase2 showed that it was blocked, internal amino acid sequences were analyzed after CNBr cleavage treatment of purified APase2 and separation of the cleaved peptides by Tricine/SDS-PAGE. The treatment gave two major peptides of 10 and 8 kDa, and amino acid sequencing showed that the N-terminus of a 10-kDa peptide was blocked and the sequence of an 8-kDa peptide was "KLISVKINAE KLKEGDYYLD". A similarity search of Swissplot and translated EMBL databases suggested that the sequence was highly similar to a putative APase N of *Arabidopsis thaliana*[18] and similar to internal amino sequences of APase N of microorganisms[19,20] (Fig. 3). Thus, from these results, we conclude that purified APase2 belongs to APase N (clan MA, family M1).

Origin	Sequence (region)		Reference
Cucumber APase2	KLISVKINAE KLKEGDYYLD		This study
Arabidopsis APase N	KLLSVKVEGK KLKEGDYQLD	(137-156)	(18)
E. coli APase N	KLVSVHNDE PWTAWKEEEG	(60-79)	(19)
Neisseria APase N	KLLSVKINGA AADYVLEGET	(56-75)	(20)

Fig. 3. Internal Amino Acid Sequence Alignment of APase2 and Similar Proteins of Other Organisms.

Amino acid sequences of an 8-kDa peptide and similar proteins are shown. Accession numbers of amino acid sequences of the similar proteins are AAG52429 (*A. thaliana*), AAA24318 (*E. coli*), and AAF41777 (*Neisseria meningitidis*). Identical amino acid residues are shown in filled boxes.

Plant APases found so far can be divided into two classes on the basis of their biochemical properties.[4,9] The first group comprises monomeric enzymes with a molecular mass of 60 to 90 kDa and pH optimum at neutral pH. Most plant APases purified from germinating seedlings belong to this group.[1,3] Another group contains large, 150–300 kDa, metalloproteases that have alkaline pH optima.[4,9] The biochemical properties of cucumber APase N suggest that it belongs to the latter group.

Many plant APases have so far been studied, however the only APases identified molecular biologically are the Leu-APases of *A. thaliana* and tomato.[9,10] These Leu-APases are metalloproteases of high molecular weight like cucumber APase N, however, the amino acid sequences are evidently distinct from cucumber APase N. Recently, an Ala-pNa cleaving APase was purified from mesquite pollen and its partially analyzed amino acid sequence was similar in sequence to APase N of *E. coli*.[21] Mesquite and cucumber APases belong to the same class of plant APase N because both of the amino acid sequences show high similarity to a putative APase N of *A. thaliana*, although the known amino acid sequences of mesquite and cucumber APases do not overlap.

APases of plants play important roles in many biological processes.[1-7] A well-documented class of plant APases, Leu-APases, have been implicated in wound healing and plant defence response.[5,22] mRNAs for Leu-APase were barely detectable in healthy plants and accumulated to high levels in response to infection, insect infestation, and mechanical wounding. On the other hand, although the detailed expression mechanism of plant APase N is unknown,

APase N might be mainly involved in the general turnover of proteins and peptides essential for cell maintenance in plant tissues because it is highly active in healthy plant tissues and has broad substrate specificity.

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