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Note

Purification and Characterization of Purple Acid Phosphatase PAP1 from Dry Powder of Sweet Potato

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Purple acid phosphatase (PAP) was purified from sweet potato dry powder, which is used as a food additive. Spectrometric and enzymatic analyses, and analysis of the amino-terminal sequence indicated that the purified purple acid phosphatase was PAP1. High activity in neutral and acidic conditions, broad substrate specificity, and good thermal stability of PAP1 suggest the possibility of practical applications of PAP1.

Key words: purple acid phosphatase (PAP); sweet potato PAP1; dry powder of sweet potato; binuclear Fe–Mn center

Purple acid phosphatases (PAPs) are widely distributed in animals, plants, and microorganisms, and catalyze the hydrolysis of activated phosphoric acid ester and phosphoric acid anhydrides in the pH range 4–7. Recently, the reaction mechanism of PAP from kidney beans was proposed on the basis of its crystal structure.[1] This enzyme has a binuclear Fe(III)–Zn(II) center, and shows specifically high activity toward ATP as an ATPase. In contrast, PAP from sweet potato has a binuclear Fe(III)–Mn(II) center.[2] Unfortunately, however, the tertiary structure of PAP from sweet potato has not been identified yet. Comparison of tertiary structure of PAP between sweet potato and kidney beans would reveal the structure-function relationship of the PAPs and their physiological roles. A convenient method for purification of PAP would allow us to obtain a large amount of pure PAP for crystallization. In this study, we attempted to establish a highly convenient and reproducible method for purification of PAPs from a dry powder of sweet potato.

In order to examine the multiplicity of PAP in the materials, non-denaturing PAGE followed by activity staining was done as follows. Fresh sweet potato tissue was quickly frozen in liquid nitrogen, ground in a cold mortar, and suspended in 1.0 ml 0.1 M acetate buffer (pH 6.0). After centrifugation of the suspension at 10,000 × *g* for 10 min at 4°C, the supernatant was put on a non-denaturing 4 to 20% linear gradient polyacrylamide gel at 4°C. After staining with Fast Blue RR and α-naphthyl phosphate, four distinct bands were observed on the gel in fresh sweet potato (data not shown). On the other hand, only one major band was detected in the dry powder, suggesting that only one isoform of the acid phosphatases retained the activity during the processes for production of dry powder.

The purple acid phosphatase was purified as follows. The sweet potato dry powder (250 g) was suspended in 500 ml of 50 mM acetate, pH 5.6, containing 0.5 M NaCl, and filtered through filter paper to yield a crude extract. After addition of ammonium sulfate at 25% saturation, the crude extract was centrifuged at 10,000 × *g* for 30 min. The supernatant was applied to a Toyopearl phenyl-650M column (39 mm I.D. × 17 cm) equilibrated with the same buffer. The column was washed with 1 l of equilibration buffer and eluted with a linear gradient of ammonium sulfate (1.0–0.5 M). The eluent containing acid phosphatase was dialyzed against 5 L of 50 mM acetate, pH 5.6, and put on a DEAE Affi-gel Blue (27 mm I.D. × 19 cm) equilibrated with the same buffer. After washing the column with 5 volumes of the same buffer, the acid phosphatase was eluted with a linear gradient of NaCl (0–1.5 M). The fraction containing acid phosphatase was dialyzed against 50 mM acetate, pH 5.6, containing 0.5 M NaCl, and applied to a Con A Sepharose 4B column (16 mm I.D. × 6 cm) equilibrated with the same buffer. The column was washed with 10 column volumes of the same buffer, and eluted with a linear gradient of glucose concentration (0–0.2 M). The fraction containing acid phosphatase was concentrated by ultrafiltration (Amicon YM-30) and put through gel permeation chromatography on a Sephacryl S-200 HR column (16 mm I.D. × 55 cm) equilibrated with 50 mM acetate, pH 5.6, containing 0.5 M NaCl. Table I summarizes the purification of the acid phosphatase. The specific content of acid phosphatase of the crude

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Abbreviations: PAP, purple acid phosphatase; ALP, alkaline phosphatase; pNPP, p-nitro-phenyl phosphate

juice from the dry powder was nearly the same as that from fresh sweet potato (data not shown). SDS-PAGE analysis under reducing conditions indicated that the extract of the dry powder contained β-amylase with a molecular weight of 53,000 and a protein with a molecular weight of 24,000 as major proteins (Fig. 1). The eluent from the DEAE Affi-gel blue column chromatography showed a clear band corresponding to purple acid phosphatase (Fig. 1). The specific activity was dramatically increased after DEAE Affi-gel blue column chromatography. Con A-Sepharose and Sephacryl S-200 column chromatography also increased the specific activity. The final sample showed the specific activity of 609 U/mg protein, which was nearly the same as that reported by Hefler *et al.*[3] The mean ± SD value of the specific activity of the purified PAP1 was calculated to be 608 ± 20 U/mg protein from five separate experiments. SDS-PAGE analysis under reducing conditions demonstrated that a molecular mass of the purified acid phosphatase was 60,000. On the other hand, gel permeation chromatography with Sephacryl S-200 HR indicated that the purified acid phosphatase had a molecular mass of 120,000. These results strongly suggest that the purified acid phosphatase exists as a dimer as described by Uehara *et al.*[4] The purified acid phosphatase was a purple color and its absorption spectrum had an absorption maximum at 550 nm. The A280/550 ratio was calculated as 30, in good agreement with the previous studies.[4,5] The N-terminal sequence of the purified PAP was LPNAEDVDMPWVS, which was identical with that of PAP1.[2] The advantages of the dry powder for the purification of PAP are summarized as follows. First, dry powder is much more stable than fresh sweet potato, and can be used readily all the year. Secondly, a good reproducibility with a good yield was obtained on the purification of the acid phosphatase. Thirdly, the dry powder appears to contain a single form of acid phosphatase, PAP1, although fresh sweet potato contained multiple forms of acid phosphatase.

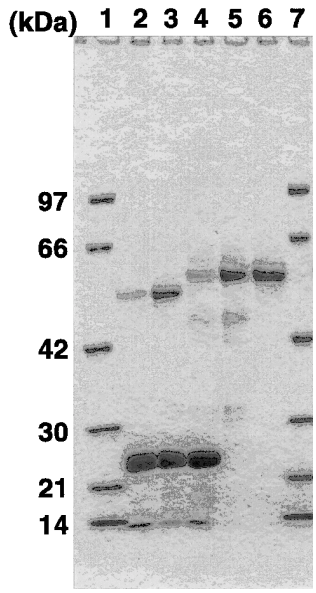

Fig. 1. SDS-PAGE Analysis of the Purified Samples from Each Step.

Linear gradient polyacrylamide gel (4–20%) from Daiichi Pure Chemicals (Tokyo, Japan) was used. Lanes 1 and 7 molecular markers (rabbit phosphorylase, Mw. 97,400; bovine serum albumin, M_r 66,267; rabbit aldolase, M_r 42,400; bovine carbonic anhydrase, M_r 30,000; soy bean trypsin inhibitor, M_r 21,000; egg white lysozyme, M_r 14,400); lane 2, crude extract; lane 3, eluate from TOYOPEARL phenyl-650M; lane 4, eluate from DEAE Affi-gel blue; lane 5, eluate from Con A-Sepharose 4B; lane 6, eluate from Sephacryl S-200. Each well of lanes 2–6 contained 1.0 μg of protein.

For the measurement of phosphatase activity toward pNPP, the amount of *p*-nitrophenol was estimated on the basis of the absorbance change at 410 nm. The phosphatase activity toward glucose-6-phosphate, fructose-6-phosphate, sucrose-6-phosphate, ATP, ADP, AMP, and NADP was estimated by measuring the phosphate concentration as described by Heinonen and Lahti.[6] The kinetic constants K_m and k_{cat} were calculated by nonlinear regression analysis using the Kaleida-Graph (Synergy Software, Readings, PA). Table II summarizes the phosphatase activity of the purified PAP1 toward 8 substrates. Among the substrates, the purified PAP1 showed the lowest K_m and the highest k_{cat} toward pNPP at pH 5.6. The PAP1 showed similar k_{cat}/K_m toward glucose 6-phosphate and fructose 6-phosphate, while it showed a significantly lower value toward sucrose 6-phosphate. Among ATP, ADP, and AMP, PAP1 showed the lowest K_m toward ADP and the highest k_{cat} toward ATP. Judging from the k_{cat}/K_m, NADP was considered to be a better sub-

Table 1. Purification of PAP1 from Sweet Potato Dry Powder

Step and fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)	Recovery (%)
Crude juice	2200	4276	1.9	1	100
TOYOPEARL Phenyl-650M	452	2419	5.3	3	60
DEAE Affi-gel Blue	17	1996	117	60	42
Con A Sepharose 4B	5.5	1669	301	155	37
Sephacryl S-200 HR	2.2	1335	609	313	29

Table 2. Substrate Specificity of the Purified PAP1

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
p-NPP	531	0.21	2528
Glucose-6-phosphate	218	2.17	100
Fructose-6-phosphate	171	1.93	88
Sucrose-6-phosphate	35	1.27	27
ATP	155	1.50	103
ADP	50	0.53	95
AMP	45	0.83	53
NADP	295	0.97	303

strate than ATP. The high activity toward NADP indicates the possibility of application of PAP1 to a synchronous enzyme-reaction system described in our previous report.[7] The substrate specificity of PAP1 observed in this study was nearly the same as that of PAP reported previously.[2,5]

Mammalian and bacterial alkaline phosphatases (ALPs) are used in immunoassays and DNA modification. However, the activity of ALP at neutral pH is much lower than that at optimal pH. In order to examine the possibility of practical applications of the purple acid phosphatase, we compared the enzymatic properties and thermal stability of the purified PAP1 with those of calf intestine ALP, which showed its maximum activity at pH 10.5. The kinetic parameter k_{cat} of PAP1 for pNPP at pH 7.0 was 377 s^{-1}, while the k_{cat} of ALP at pH 7.0 was 93 s^{-1}. Thus, the k_{cat} of PAP1 was 4 times higher than that of ALP at pH 7.0. To examine temperature dependence of the activity, PAP1 or calf intestine ALP was added to 0.1 M Bis-Tris-HCl (pH 7.0) containing 5.0 mM pNPP incubated at each temperature. PAP1 and ALP showed the highest activity at 50°C and 60°C, respectively. Based on Arrhenius plots in the temperature range 25–50°C, the activation energy of PAP1 and ALP was calculated to be 41.6 and 49.3 kJ/mol, respectively. To examine the thermal stability of PAP1 and calf intestine ALP, the activity was measured at 25°C, after incubation for 30 min in 0.1 M Bis-Tris-HCl (pH 7.0) at 40°C and 55°C. No loss of the activity was observed at 40°C for either PAP1 or ALP. The residual activity of PAP1 and ALP at 55°C was 60 and 69%, respectively, suggesting that the thermal stability of PAP1 is not very different from that of ALP. These results strongly suggest the possibility of practical applications of PAP1, for example, an enzyme-linked immunoassay using enzymes that are active in neutral or acidic conditions.

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