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60 kD POLYPEPTIDE OF CELL WALL ACID PHOSPHATASE FROM TOBACCO CELLS

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Abstract—The native cell wall acid phosphatase (APase), WP-II, of tobacco XD-6 cells was purified by chromatographic procedures. The purified WP-II yielded one major band of M_r of 60 kD on SDS-polyacrylamide gel electrophoresis(PAGE) after SDS treatment at 100°C for 5 min. The preparation, however, yielded an additional smear band with M_r of 120 kD that retained phosphatase activity after the treatment with SDS at 20°C for 3 hr. Two dimensional SDS-PAGE with different denaturation suggested that the 120 kD band consisted of the 60 kD polypeptide. Renaturation of the 60 kD polypeptide recovered about 30% of the activity of the crude WP-II and 8% of that of the purified WP-II, respectively.

INTRODUCTION

The native cell wall acid phosphatase (APase, EC 3.1.3.2) is an intrinsic component of plant cell walls and is useful in the study of protein trafficking mechanisms to the cell surface. In a previous paper we showed for the first time the purification and some properties of the native cell wall APase of tobacco cells [1]. The native cell wall APase fraction was eluted at 0.6 M NaCl from a column of isolated cell walls of tobacco cells with a linear gradient of NaCl. A 48-fold purification was obtained by protein fractionation with hydroxyapatite, DEAE-Sephacel and butyl-Toyopearl 650. Native electrophoresis showed a diffuse protein band corresponding to the enzyme activity. It was suggested that the enzyme was purified as a homogeneous enzyme protein.

Recently we reported that tobacco protoplasts secreted two isoforms of APase during regeneration of the cell wall [2]. Those APases are important in the regeneration of plant cell walls from protoplasts, and knowledge of trafficking to the cell surface and involvement in cell wall assembly is important in understanding the regeneration process.

In order to study mechanisms of trafficking cell surface protein and cell wall assembly we aimed to isolate and purify the active polypeptide of the native cell wall APase(WP-II) which was eluted from cell walls with 0.7 M NaCl. The ability of recovering enzyme activity after a protein has been electrophoresed on SDS-polyacrylamide gels is useful for correlating an enzyme activity with a particular protein band on the gel and to obtain small amounts of very pure protein. In this study we improved the method of purification of the enzyme to obtain higher recovery and specific activity. We therefore determined the recovery of the polypeptide with WP-II by the method described previously [3] with modification.

RESULTS AND DISCUSSION

Purification of the cell wall APase (WP-II) of tobacco cells

The APase from cell walls of tobacco XD-6 cells (designated as WP-II) was purified by the method described previously [1] with modifications. WP-II was extracted by 0.7 M NaCl. A suitable procedure for purification of WP-II was chromatography on hydroxyapatite, followed by chromatography on butyl-Toyopearl 650 (Ist) and rechromatograpny on butyl-Toyopearl 650 (IInd). On hydroxyapatite column chromatography the enzyme elution was chro-

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Table 1. Summary of purification procedure

Step	Protein (mg)	Activity (nkat)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
Crude extract	38	119	3.1	100	1.0
Hydroxyapatite	7.3	70	9.5	59	3.1
Butyl-Toyopearl (I)	0.28	43	152	36	49
Butyl-Toyopearl (II)	0.15	27	179	23	57

matographed in the following two steps: first with 250 mM Tris-HCl and second with 3 M Tris-HCl, pH 7. About 60% of the enzyme activity of the crude WP-II was recovered in the eluate of 3 M Tris-HCl (Table 1). Further purification was achieved by chromatography on butyl-Toyopearl 650 (Ist) instead of DEAE-Sephacel previously used [1]. The butyl-Toyopearl 650 column chromatography was effective and the recovery of the enzyme activity was about 60% of the hydroxyapatite fraction (Table 1). Since the recovery of the enzyme activity in the DEAE-Sephacel was only about 5%, as reported previously [1], this modification provided great improvement for purification of the enzyme. The APase activity was then purified by rechromatography on butyl-Toyopearl 650 (IInd). A typical elution profile obtained in this step is shown in Fig. 1. The recovery of WP-II was *ca* 23% and the purification of the enrichment of *ca* 58-fold (Table 1). The purified WP-II yielded one major band, the M_r of the subunit was 60 kD from the position of the band on the SDS-PAGE gel (Fig. 2).

Protein with the WP-II is a 60 kD polypeptide

Purified WP-II preparation yielded a 60 kD polypeptide on SDS-PAGE if it was treated with SDS sample buffer at 100° for 5 min(Fig. 2, a, b), but the peptide band did not show phosphatase activity on the gel (Fig. 2, e). However, the pattern of the proteins changed if the same preparation was treated with SDS sample buffer at 20° for a few min to 3 hr. In this case, a smear polypeptide band with M_r of 120 kD appeared with concomitant decrease of the 60 kD polypeptide (Fig. 2, c, d). Moreover, the 120 kD polypeptide showed strong phosphatase activity on the gel, when it was stained for phosphatase activity by use of α-naphthyl phosphate and Fast Garnet GBC salt (Fig. 2, f and g). The 120 kD polypeptide was consequently regarded as a resistant part of the WP-II to the SDS

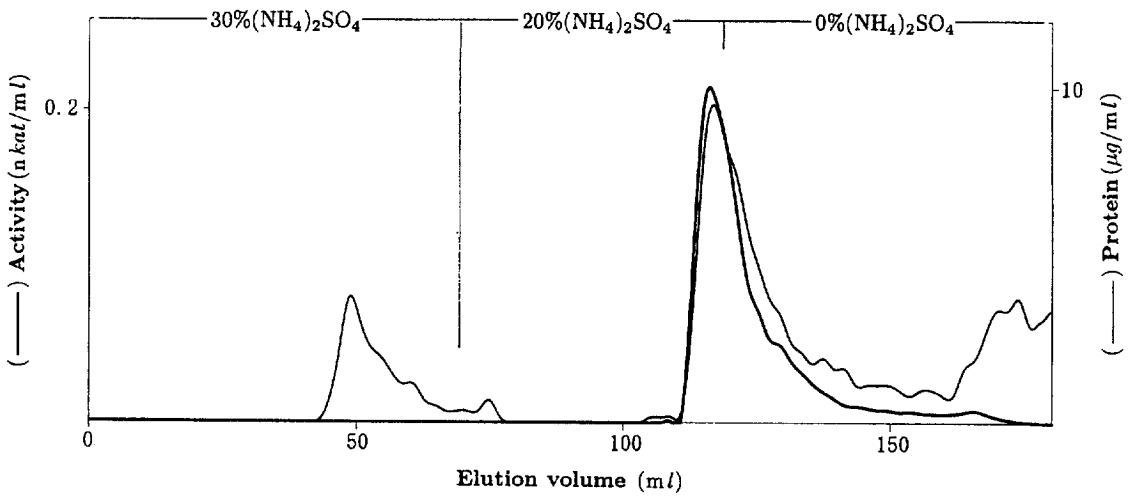

Fig. 1. Elution profile of acid phosphatase activities from Butyl-Toyopearl 650 column chromatography(II). High activity fractions of Butyl-Toyopearl 650 column chromatography(I) of WP-II was applied to Butyl-Toyopearl 650 column (II)(2 × 20 cm) previously equilibrated with 10 mM Tris-HCl containing 80% saturated ammonium sulfate (pH 7). Elution was developed first with 70 ml of the equilibration buffer containing 30% saturated ammonium sulfate, at a flow rate of 3 ml per h. Second, elution was developed with 50 ml of the same buffer containing 20% saturated ammonium sulfate and third with 60 ml of the same buffer, at a flow rate of 1 ml per min. Fractions of 2 ml of each were collected and aliquots of the eluates were assayed for hydrolytic enzyme activity.

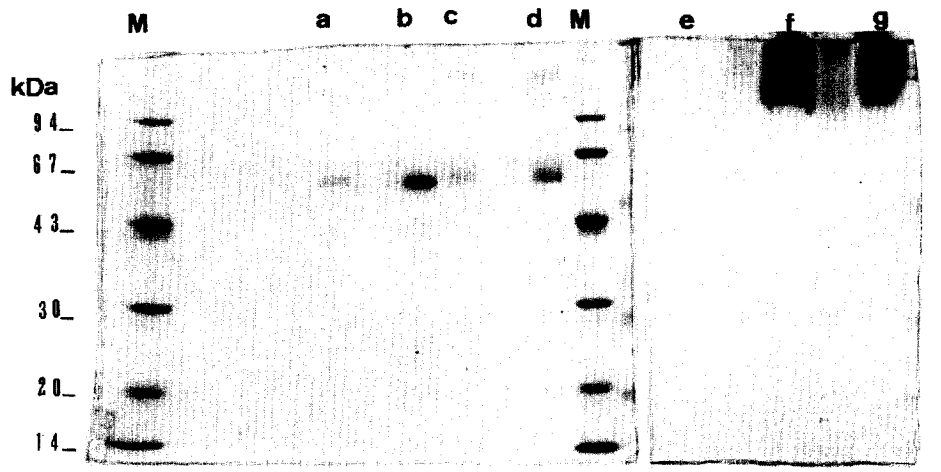

Fig. 2. SDS-PAGE analysis of tobacco WP-II. Purified WP-II was subjected to SDS-PAGE after heating with sample buffer at 100° for 5 min(lanes **a**, 1.3 μg; **b**, 2 μg; **e**, 2 μg) or at 20° for 3 hr (lanes c, 1.3 μg; **d**, 2 μg; **f**, 1.3 μg; **g**, 2 μg). After electrophoresis the gel was stained for protein (lanes **a**, **b**, **c**, **d**), and for APase activity (lanes **e**, **f**, **g**). M_r standard: lane **M**(2 μg)

treatment. Interestingly, the molar ratio of the 120 kD and the 60 kD polypeptide did not change with time of incubation.

In order to identify proteins in the 120 kD polypeptide region, the WP-II fraction was analysed by two-dimensional PAGE. The WP-II fraction (5 μg protein) was treated with SDS sample buffer at 20° and the sample was immediately subjected to SDS-PAGE (first dimension). The lane of the first dimensional separation was cut out, soaked in SDS sample buffer for 20 min, heated in boiling water bath for 5 min, and subjected to second dimensional SDS-PAGE by use of a slab gel. The result is shown in Fig. 3.

As shown in Fig. 3, two dimensional PAGE clearly indicates that the polypeptides that migrated to 60 kD and 120 kD in the first dimension, migrated to the same M_r of 60 kD in the second dimension. It is likely when the 120 kD polypeptide, obtained in the first dimensional PAGE, was denatured by SDS at 100° for 5 min, it decomposed into the enzymatically inactive 60 kD polypeptide. These results strongly suggest that the major subunit of the WP-II is a 60 kD polypeptide (Fig. 3). The M_r of native WP-II was found by HPLC gel filtration to be about 70 kD relative to the standard markers (data not shown), and it is suggested that the native form of WP-II is not a homo-dimer but a single homogeneous protein. This is consistent with the previous observation [1] that the purified WP-II preparation was a single homogeneous protein, co-migrating with the APase activity in gel electrophoresis under non-denaturating conditions.

Renaturation of the 60 kD polypeptide

In order to determine whether the 60 kD polypeptide was the catalytic protein, we tried renaturation of the polypeptide according to the methods described in ref [3]. Buffer conditions for optimal renaturation was primarily examined, using crude WP-II as enzyme source. Crude WP-II was precipitated by acetone with casein as a carrier protein, dissolved with 6 M guanidine-HCl in a buffer, diluted rapidly in the same buffer, and stood at room temp. for 24 hr. Only Buffer A, containing Murashige and Skoog's basal major salts, pH 5.7, 0.15 M NaCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 10% (w/v) glycerol and 10% (w/v) polyethylene glycol, was effective for renaturation of the crude WP-II, and about 31% of the final recovery of activity was obtained. No other buffer such as Tris-HCl, pH 7.5 was effective. Incubation at 4° was much less effective than at room temp. (20°) (data not shown). These data suggest that the purified WP-II requires physiological conditions for optimal renaturation, since the tobacco cells are cultured at 25° in the medium in which Murashige and Skoog's basal major salts are the major constituent. Recovery of phosphatase activity in BufferA increased with time and reached a maximum of about 30% at 24 hr reproducibly (refer Table 2), but it decreased gradually thereafter (data not shown). On the other hand, Mn^{2+} did not show any effects for renaturation. This suggested that the tobacco WP-II is not a metallo-enzyme, though some plant APase are known to be metallo-enzymes which requires Mn^{2+} as a co-factor [8, 9]. In contrast, another refined method for

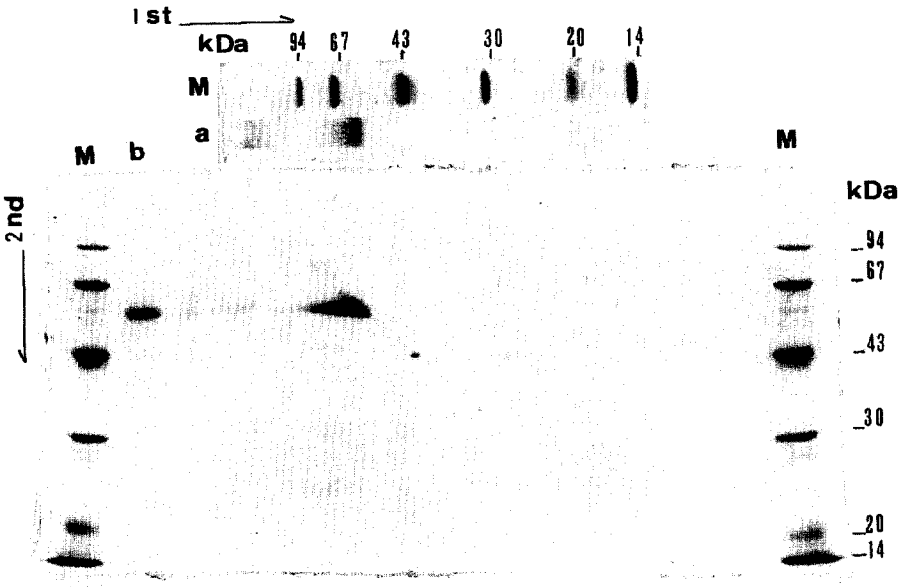

Fig. 3. Patterns after two-dimensional gel electrophoresis of WP-II. A preparation of WP-II (5 μg/lane) was separated by SDS-PAGE in the first dimension, immediately after it was mixed with SDS at 20°. The gel strip was cut out, and soaked in SDS-sample buffer for 20 min, incubated with SDS at 100° for 5 min, transferred to a second dimensional SDS-PAGE in a slab gel (lane **a**) and electrophoresed. The other preparation of WP-II was applied to the gel after heating with SDS buffer at 100° for 5 min as a control (lane **b**). *M*, standard: lane **M**(2 μg).

renaturation [10] was not effective for the purified WP-II.

Purified WP-II preparation also showed phosphatase activity by the renaturation procedure, but only about 8% recovery was obtained even in the optimal renaturation conditions (Table 2). Furthermore, renaturated phosphatase activity was also demonstrated for the 60 kD polypeptide separated by SDS-PAGE. No other part of the gel showed phosphatase activity in the purified WP-II fraction, but an additional polypeptide band that showed renaturated phosphatase activity was found in a crude WP-II fraction (data not shown). We suggest from these data that the 60 kD polypeptide is the enzymatically active component of the WP-II. However, the recovery of activity in the 60 kD polypeptide band was even lower than that of purified WP-II fraction and was often less than 1%. This would be partly due to very low recovery of extraction of the 60 kD polypeptide from the gel (data not shown). But low recovery of renaturation especially for purified preparations was possibly due to loss of co-factor of the enzyme or factors that are needed for effective renaturation of the polypeptide.

Table 2. Recovery of enzyme activity after renaturation

Enzyme	Time after dilution from guanidine-HCl	Activity recovery (%)
Crued WP-II	16 hr	13–17
	24 hr	30–33
WP-II	24 hr	7–9

EXPERIMENTAL

Plant material and culture method

Tobacco (*Nicotiana tabacum* L. var Xanthi) cell line XD-6 was used. Cells (*ca* 5g fr. wt) grown in late log phase were transferred to a 100 ml flask containing 40 ml modified Murashige and Skoog's medium [4]. The flask was shaken on a reciprocal shaker (5 cm amplitude) operating 110 times per min at 25° under dim light in day time for 10–14 days. Cells (*ca* 30 g fr. wt) were collected by filtering off the suspension and washed with 500 ml deionized H_2O.

Preparation of cell walls

Cell walls were prepared by the method described previously [5] with modification [1].

Enzyme extraction and purification were carried out by the methods described previously [1] with modification. All steps were carried out at 0–4°. Chromatographic procedures were monitored for protein at 280 or 230 nm. The cell walls were frozen at −20° for 2 days and then thawed and washed twice with deionized H_2O. The freeze-thawed cell walls were again frozen at −20° for 2 days. Cell walls (*ca* 20 g fr. wt) were then suspended in 200 ml of 10 mM Tris-

HCl, pH 7, containing 0.4 M NaCl and stirred gently at 4°. After 30 min, the suspension was centrifuged at 7500 *g* for 30 min. The cell walls were suspended in 200 ml of 10 mM Tris-HCl, pH 7, containing 0.7 M NaCl and stirred gently at 4°. After 30 min, the suspension was centrifuged at 7500 *g* for 30 min. The supernatant was concd with sucrose and was called 'crude WP-II'. After dialysis against 250 mM Tris-HCl, pH 7, the crude WP-II extract was applied to a hydroxyapatite column (2 × 16 cm). The column was washed with 250 mM Tris-HCl, pH 7(120 ml) and the enzyme was eluted with 3 M Tris-HCl, pH 7. Fractions with enzyme activity were pooled and concd with sucrose. To the active fractions was added solid $(NH_4)_2SO_4$ to give 80% satn. The enzyme was loaded onto a hydrophobic interaction chromatography column of butyl-Toyopearl 650(I) column (2 × 8 cm, Toso Corporation, Japan) equilibrated with 10 mM Tris-HCl, pH 7, containing 80% satud $(NH_4)_2SO_4$. Stepwise elution was then performed by decreasing the concn of $(NH_4)_2SO_4$ contained in 10 mM Tris-HCl, pH 7. To the active fractions was added solid $(NH_4)_2SO_4$ to give 80% satn. The enzyme was loaded onto a butyl-Toyopearl 650(II) column and it was rechromatographed on the column by the method described above. Fractions with enzyme activity were pooled and concd with sucrose. The final fraction was dialysed against 10 mM Tris-HCl, pH 7, which was called WP-II.

Calibration of M_r of the WP-II

The purified WP-II was filtered on a gel filtration chromatography column of Protein pak 300 column [7.8 × 300 mm, fractionation range (10 kD–500 kD), Waters Corporation, Massachusetts, U.S.A.] using 50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl at a flow rate of 1 ml/min. The proteins used to calibrate the M_r were: glutamate dehydrogenase (290 k), lactate dehydrogenase (142 k), enolase (67 k), and adenylate kinase (32 k), cytochrome C (12.4 k), all obtained from Oriental Yeast Co. Ltd. (Japan).

Enzyme assay

The standard incubation mixture consists of 3.3 mM *p*NPP, 67 mM NaOAc buffer, pH 5.6, and 0.25 ml of enzyme in a total vol. of 0.75 ml. After the incubation period of 30 min at 35°, the reaction was terminated by adding 0.75 ml of 2 M Na_2CO_3 and the amount of *p*-nitrophenol released was determined from its *A* at 405 nm.

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was performed on 10.5% (w/v) polyacrylamide slab gels according to the method of ref.[6]. Electrophoresis samples were previously heated at 100° for 5 min in an SDS sample buffer containing 0.9% (w/v) SDS, 0.06 M Tris-HCl, pH 6.8, 9% (v/v) glycerol, 0.02% (v/v) 2-mercaptoethanol and 0.0006% (v/v) bromophenol blue. After electrophoresis, proteins were stained with Coomassie brilliant blue(CBB), Amido Black 10 or by silver staining [7]. The bands corresponding to APase were localized by means of their enzymatic activity using Fast Garnet GBC salt and α-naphthyl phosphate (2 Na) as substrate. The M_r of the subunit of purified enzyme in the presence of SDS was estimated by comparing its relative mobility with those of standard proteins of known M_r (Pharmacia electrophoresis calibration kit), phosphorylase b (94 k), bovine serum albumin(67 k), carbonic anhydrase (30 k) and soybean trypsin inhibitor (20 k) and α lactalbumin(14 k).

Two dimensional SDS-polyacrylamide gel electrophoresis(PAGE) was carried out using a gel strip of slab SDS-PAGE used as the first-dimensional gel electrophoresis.

Renaturation of the 60 kD polypeptide was performed according to the methods described in ref [3]. with modification. Crude WP-II, purified WP-II and 60 kD polypeptide separated by SDS-PAGE were used as enzyme source. Crude WP-II and purified WP-II were previously heated at 100° for 5 min in the SDS sample buffer. After SDS-PAGE of the 60 kD polypeptide, the gel was rinsed with H_2O and the CBB-stained protein bands of the gel were previously cut out into 1 mm gel pieces with a razor blade. Crude WP-II, the purified WP-II and the 60 kD polypeptide in the gel pieces were extracted for 16 hr at 37° by a buffer containing 0.05 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 0.15 M NaCl. The gel pieces were removed by centrifugation at 10 000 *g* for 10 min. Sixty μg of casein was added as carrier to the solns and polypeptides were treated with 4 vol. of acetone and allowed to ppt at −80° for 10 min. Ppts were recovered at 30 000 *g* for 10 min, and air dried. The ppts were dissolved thoroughly with 6 M guanidine-hydrochloride (guanidine-HCl) in a renaturation buffer and stood at room temp. for 30 min. The solns were then rapidly diluted 50-fold with the same renaturation buffer and stood at 4° or room temp. to permit renaturation. The most effective renaturation buffer was Buffer A, containing Murashige and Skoog's basal salts [4], 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.15 M NaCl, 10% (w/v) glycerol and 10% (w/v) polyethylene glycol. Renaturation was monitored by the phosphatase activity as described above.

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