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Characterization of Potato Polyphenol Oxidase Purified by *p*-aminobenzoic Acid-sepharose Affinity Column

Seul Ki Kim, Ho-Joon Kang, Jae-Joon Kim, and Woo-Yeon Kim*

Department of Biotechnology, Chung-Ang University, Anseong 456-756, Korea

Abstract. Polyphenol oxidases (PPO) are copper-containing enzymes responsible for tissue browning in fruits and vegetables including potato, apple and pears. Although these enzymes have been studied for many years, their physiological roles in plants are not yet clear. Therefore, these enzymes need to be purified to characterize further from potato tubers. The classical methods used for the purification of PPO involve several steps. So in this study, we developed a one-step chromatography process for the potato tuber PPO purification. After removal of salts from dissolved ammonium sulfate precipitates of potato tuber extracts using Sephadex-G50 gel filtration, affinity chromatography was carried out on NHS-activated Sepharose 4B using *p*-aminobenzoic acid as a ligand. The purified enzyme was confirmed by silver staining and a zymogram. The optimum temperature and pH for the purified potato tuber PPO were 15℃ and pH 6.0, respectively. The results obtained in the present study will aid to evaluate PPO from various fruits and vegetables.

***Additional key words*:** PABA-sepharose, silver staining, zymogram

Introduction

Polyphenol oxidase (PPO) is the major cause of enzymatic browning reaction in most plants. Quality of fresh produce has often been hampered due to PPO activity upon wounding and microbial penetration into tissues during storage and marketing. The enzyme catalyzes one or two reactions involving oxygen (Bartlett et al., 1972; Pathak et al., 1992). The first type of reaction is the *ortho*-hydroxylation of monophenols (monophenolase reaction). The second type of reaction (diphenolase reaction) is the oxidation by molecular oxygen of *o*-diphenols to o-quinones. The quinone products polymerize and react with amino acid groups of cellular proteins, resulting in a darkened pigment deposits (Thygesen et al., 1995). In higher plants PPO is present in plastids of fruits and vegetables (Mayer, 1987). Even though enzymatic browning is the cause of decrease in sensory properties and nutritional value (Porta, 1988), the function of PPO in plants remains unclear. It may be involved in the host defense mechanism and biosynthesis of plant components, and may also mediate photosynthetic electron transport (Heimdal et al., 1994). In order to study its structure and function, purified enzyme is needed. However, classical methods used for the purification of PPO from potato tubers, which are the main source of potato chips, involves DEAE-cellulose, gel filtration, and phenyl-Sepharose amongst other resins. Conventional methods consist of many steps with a low quality yield of purified enzyme (Kwon and Kim., 1996; Marri et al., 2003; Partington and Bolwell, 1996).

There are two difficulties involved in the purification of PPO from potato tubers. First is the presence of large amounts of patatin, which is the major soluble glycoprotein of potato tubers (Partington and Bolwell, 1996), and PPO and patatin are similar in molecular weight. Second is the huge amount of phenolic compounds observed (Wissemen and Montgomery, 1985). Reaction of phenolics or quinones with the enzyme can inactivate or modify the protein through covalent, hydrogen, hydrophobic, and ionic binding (Loomis, 1974). In particular, compounds such as quinones may react covalently with the enzyme, resulting in discoloration and aggregation of PPO during extraction (Kwon and Kim, 1996). Therefore, the isolation and purification of PPO from plant sources such as potato tubers is not a trivial task. Various molecular weights have been reported (Flurkey and Inlow, 2008; Kwon and Kim, 1996; Partington and Bolwell, 1996), most likely due to patatin contamination, degradation, and glycosylation of PPO. In this study, we purified the PPO from potato tubers without patatin using a PABA-Sepharose affinity column and

***Corresponding author:** wykim@cau.ac.kr

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characterized the purified PPO in terms of optimum temperature and pH.

Materials and Methods

Materials

Potato (*Solanum tuberosum* L. cv Sumi) was obtained from the local market in Korea and stored at 4℃. N-hydroxysuccinimide (NHS)-activated Sepharose 4B and Sephadex-G50 were purchased from GE Healthcare (Uppsala, Sweden). Miracloth was purchased from Calbiochem (San Diego, CA, USA). Ammonium sulfate, L-DOPA (L-3,4-dihydroxyphenylalanine), L-ascorbic acid, *p*-aminobenzoic acid sodium salt, and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Preparation of *p*-aminobenzoic acid-sepharose Column

Five milliliters of NHS-activated Sepharose 4B medium was washed with 60 mL of 1 mM HCl until the isopropanol was removed. After washing the medium, 2.5 mL of coupling solution was added and the slurry was stirred at room temperature for 2 hr. Coupling solution contained 10 mM *p*-aminobenzoic acid (PABA) in 0.2 M $NaHCO_3$, pH 8.3 with 0.5 M NaCl. After coupling, the non-reacted group on the medium was blocked with 5 mL of blocking solution containing 0.5 M ethanolamine, pH 8.5 and 0.5 M NaCl by stirring for 1-2 hr. After blocking, the slurry was packed into the column. Buffers such as 0.1 M Tris-HCl, pH 8.5 and 0.1 M acetate, pH 4.5 containing 0.5 M NaCl, respectively, were used for 3 consecutive washes. After washing the medium, the column was equilibrated with 25 mM Tris-HCl, pH 7.0.

Enzyme Purification

One hundred grams of potato tuber periderm was frozen in liquid nitrogen and ground in a stainless steel Waring blender until a fine powder was obtained. This powder was suspended in 400 mL of extraction buffer (25 mM Tris-HCl, pH 7.0 containing 20 mM ascorbic acid) and homogenized at 12,000 rpm for 10 min with a Polytron PT 3000 homogenizer (Kinematica AG, Swiss). The slurry was filtered by using one layer of Miracloth. The filtrate was centrifuged at 15,000 × g for 30 min in a Beckman JA-14 rotor. PPO was precipitated by the slow addition of 0.26 g of solid ammonium sulfate per mL of solution. After 30 min, the precipitate was recovered by centrifugation at 15,000 × g for 30 min. The resulting pellet was homogenized in 5 mL of 25 mM Tris-HCl, pH 7.0 containing 20 mM ascorbic acid using a Dounce homogenizer. In order to remove salts and phenolics, crude enzyme extract was applied to 50 mL of a Sephadex G-50 column previously equilibrated with 25 mM Tris-HCl, pH 7.0 and the flow rate was set at 0.5 $mL \cdot min^{-1}$. The column was eluted with the same elution buffer. The fractions which showed PPO activity were pooled and loaded onto 5 mL of a PABA-Sepharose column previously equilibrated with 25 mM Tris-HCl, pH 7.0. The column was washed with 3 bed volumes of 25 mM Tris-HCl, pH 7.0 and eluted with 2 bed volumes of the same buffer containing 0.1 M NaCl at a flow rate of 0.5 $mL \cdot min^{-1}$. All purification steps were carried out at 4℃.

Enzyme Assay

Enzyme activity was determined with L-DOPA (3,4-dihydroxyphenylalanine) as a substrate at 20℃ for 3 min by measuring the increase in absorbance at 475 nm. Unless stated, 1.4 mL of 10 mM L-DOPA in H_2O and 0.1 mL of the PPO extracts were mixed in a quartz cuvette. One unit was defined as an absorbance change during 1 min at 475 nm as described previously (Kwon and Kim, 1996).

SDS-PAGE and Protein Staining

The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis carried out according to the method of Laemmli (1970). The resolving gel was 12% acrylamide. Electrophoresis (Fisher Scientific, IA, USA) was run at 80 V for 2 hr. After the gel was cut in half, half of the gel was stained with Coomassie brilliant blue R-250 and the rest of the gel was stained with a Silver Staining kit (Invitrogen, CA, USA).

Activity Staining (zymogram) for PPO

Activity staining in the gel was performed according to the method of Angleton and Flurkey (1984) with a modification. Protein samples containing without β-mercaptoethanol were not heated, and loaded to the gel. After running, the gel was rinsed with H_2O and incubated in 10 mM L-DOPA for about 30 min. The reaction was stopped by removing the gel from the incubation solution and rinsed twice with H_2O, and then kept in destaining solution.

Characterization of PPO

To obtain the optimum pH and temperature, L-DOPA was used as a substrate. PPO activity was determined using various buffers in the pH range between 4.0 and 9.0. The buffer systems were prepared according to the method by Britton and Robinson (1931). PPO activity was determined at 5℃, 10℃, 15℃, 20℃, 25℃, 30℃, 35℃, and 40℃, respectively.

Results and Discussion

In this study, we used potato tuber periderm because it shows higher PPO activity than the rest of the tubers (Kim and Dean, 1998). In addition, we used the extraction buffer containing 20 mM ascorbic acid to prevent the browning reaction by oxidation during extraction. It has been found that 20 mM ascorbic acid inhibited the browning reaction effectively during the extraction experiment. Sephadex-G50 was used to remove small molecules including salts and phenolic compounds from the fractions containing PPO and was used to change to the buffer without ascorbic acid. There appeared to be less PPO-like bands between 42-67 kDa, which were reported to be potato tuber PPO (Flurkey and Inlow, 2008; Kwon and Kim, 1996) in the Coomassie-stained gel (data not shown). Samples that did not form aggregated complexes due to the browning reaction were helpful in the next purification step. The band between 30 kDa and 40 kDa was expected to be patatin (Partington and Bolwell, 1996). The fractions containing PPO activity were then loaded onto the PABA-Sepharose column. After loading, the column was left to stand for 10 min to aid protein binding to the resin. After washing the resin, PPO was eluted with the buffer containing 0.1 M NaCl. The fractions showing PPO activity were analyzed by SDS-PAGE (Fig. 1). As shown in the eluted fractions (lanes 7-10), most patatin was removed effectively by PABA-Sepharose column chromatography. After electrophoresis, half of the gel was stained with Coomassie briliant blue (Fig. 2A) and the other half was stained with silver (Fig. 2B). In addition, a zymogram was carried out (Fig. 2C). No PPO-like protein was observed in the case of Coomassie stained gel. However, a PPO-like protein band was observed just below 46 kDa in the silver stained gel (Fig. 2B), and PPO activity was confirmed by a zymogram (Fig. 2C).

Affinity purification was very effective because it could remove most of the patatin. We compared the different purification methods to confirm the effectiveness of affinity column chromatography (Fig. 3). DEAE-Sephadex, Sephadex G-50, and Sephadex G-100 (Urszula et al., 2007) were used for the purification PPO from the potato tubers. We measured protein content by using the gel scanning technique due to low amount of protein. The specific activity increases approximately 20-fold from 18.2 to 370 units/μg. The total activity

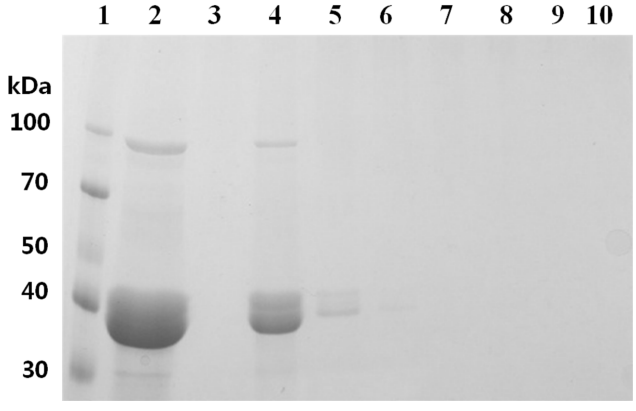

Fig. 1. SDS-PAGE of the polyphenol oxidase purified on PABA-Sepharose column: Lane 1, molecular weight marker; lane 2, an active fraction of Sephadex-G50; lane 3, flow-through; lanes 4-6, wash fractions; lanes 7-10, purified fractions. The gel was stained with Coomassie blue. Molecular weight markers are from Elpis-biotech (Daejon, Korea).

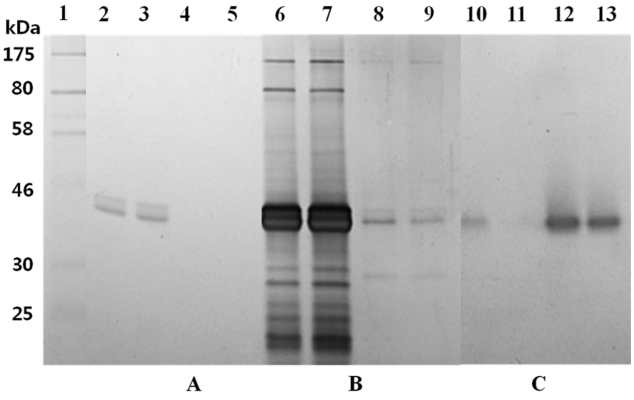

Fig. 2. SDS-PAGE, silver staining and zymogram of the polyphenol oxidase purified on PABA-Sepharose column: A, the Coomassie stained gel (lanes 1-5); B, the silver stained gel (lanes 6-9); C, the zymogram (lanes 10-13). Lanes 1, 6, and 10, 50-fold diluted active fraction from Sephadex G-50; lanes 2, 7, and 11, 8-fold diluted flow-through; lanes 3, 4, 8, 9, 12, and 13, purified fractions; lane 1, molecular weight marker. Molecular weight markers are from New England Biolabs (Ipswitch, MA.).

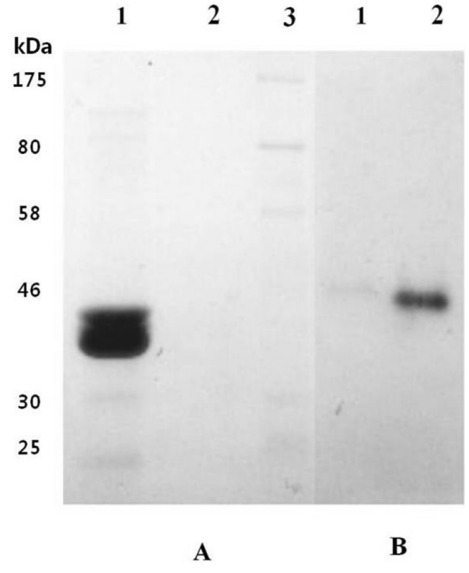

Fig. 3. SDS-PAGE for comparison with another purification method: A, the Coomassie stained gel; B, the zymogram. Lane 1, a fraction purified using by the different purification method with DEAE-Sephdex, Sephadex G-50, and Sephadex G-100 consecutively; lane 2, a fraction purified using by PABA-Sepharose; lane 3 of A, molecular weight markers.

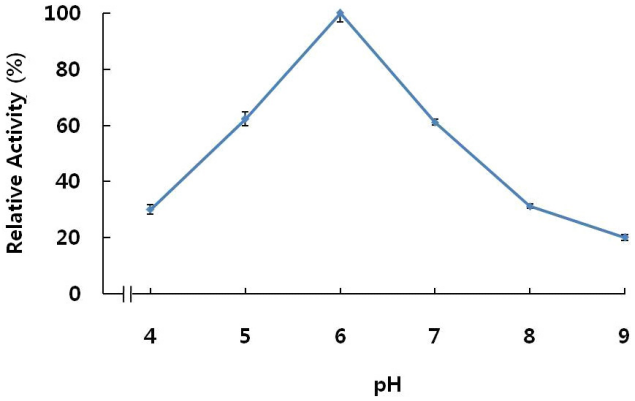

Fig. 4. Effect of pH on PPO activity. L-DOPA was used as a substrate. PPO activity was determined at 15℃ from pH 4 to pH 9.

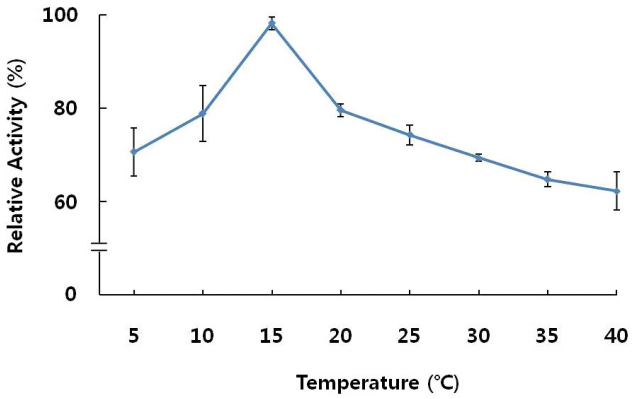

Fig. 5. Effect of temperature on PPO activity. L-DOPA was used as a substrate. PPO activity was determined at pH 6 from 5℃ to 40℃.

of the input sample was 6140 units and that of the eluted fractions was 3446 units ended up with 56% yield. Therefore, the purification was approximately 20-fold with over 50% yield on the PABA-Sepharose affinity column. The specific activity of the PPO purified by the above column was approximately 5 times higher than that of the PPO purified by a previously published method (Urszula et al., 2007) based on the scanning of the silver stained gel. Pathak and Ghole (1992) used *p*-aminobenzoic acid as a ligand of the affinity resin for the purification of potato tuber PPO previously. They used cyanogen bromide- or divynylsulfone-activated Sepharose 4B compared to the NHS-activated Sepharose 4B in our study. They showed that the purification yield was less than 20%. Pathak and Ghole (1994) also used *p*-aminophenylacetic acid as a ligand with the same coupling methods resulting in similar data to their previous report (1992). In the case of purification using PABA-Sepharose 4B activated by NHS in our study, the purification was much better than the Urszula method in terms of purification yield and degree. It might be partly due to presence of the spacer arm in the case of NHS-activated Sepharose. As shown in Fig. 4, patatin could only be removed by affinity purification (compare lanes 1 and 2 of Fig. 3). It has been reported that patatin could be completely removed by chromatography on octyl-Sepharose (Partington and Bolwell, 1996). Patatin was reported to be more hydrophobic than PPO. In this experiment the purified PPO had a molecular weight of over 40 kDa. In fact, the calculated molecular weight is between 41.8 and 57.5 kDa (Flurkey and Inlow, 2008), even though SDS-PAGE molecular weight is 60 kDa in the case of potato. The PPO purified using Con A Sepharose column was reported to be between 60 and 69 kDa (Kwon and Kim, 1996; Pathak and Ghole, 1994), which might be glycosylated forms due to the binding characteristics to Con A. The degree of glycosylation may be involved in the difference in molecular weight of PPO, even though there is debate about the molecular weight of purified and/or in vivo PPO.

An assay was carried out by reacting the purified protein in the presence of the various pH buffers and the highest activity was shown at pH 6 as shown in Fig. 4. To investigate temperature dependency, PPO activity was measured between 5℃ and 40℃. The highest activity was seen at 15℃ as shown in Fig. 5. On the zymogram, purified PPO is more active than PPO not bound to the PABA-Sepharose (Fig. 3). To investigate this phenomenon, PPO activity was measured in the presence of 0.05% SDS , which is the SDS concentration in the gel. PPO activity increased by 30% in the presence of 0.05% SDS, especially in the case of affinity column purified PPO (data not shown). In the future, the purified PPO would be helpful for the characterization of potato tuber PPO and to prevent browning of potato tubers, which is being used for the production of potato chips.

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