

## Evaluation of Several Methods for Estimation of the Total Activity of Potato Polyphenol Oxidase

A.F. HSU, C.E. THOMAS, and D. BRAUER

### ABSTRACT

Several methods of preparing homogenates from potato tuber for estimating the total activity of polyphenol oxidase (PPO) were compared. To remove endogenous phenols that may inhibit PPO, homogenates were either treated with polyvinylpyrrolidone (PVPP) or subjected to extensive dialysis, ammonium sulfate or acetone precipitation or Sephadex G-25 chromatography. The G-25 gel filtration method was superior in removing phenolic compounds and in retaining PPO activity. Using this chromatographic method, the tubers of Russett-Burbank cultivar were found to contain highest PPO activity followed by Russett and then Atlantic cultivars.

### INTRODUCTION

THE PROPERTIES of polyphenol oxidase (PPO), which have been studied extensively (Mayer and Harel, 1979; Vaughn and Duke, 1984), play an important role in browning of fresh fruits and vegetables. Despite numerous reports describing the purification of PPO (Kertesz and Zito, 1965; Loomis and Bataille, 1966; Park et al., 1980), little progress, however, has been made in the development of a rapid and quantitative procedure for the extraction of PPO from plant tissue. The purification of PPO by hydrophobic interaction chromatography is effective but may not be useful in determining the total enzyme activity in tissues because of low recovery (Weisseemann and Lee, 1980). To assay PPO activity, endogenous phenolic compounds must be removed, since oxidized phenols irreversibly inhibit PPO catalysis. Several methods have been used to separate the endogenous phenolic compounds from proteins. Phenols can be absorbed onto insoluble polyvinylpyrrolidone (PVPP) (Nagahashi, 1985). PPO can be precipitated by either cold acetone (Flurkey and Jen, 1978) or ammonium sulfate (Hsu et al., 1984) to remove phenolic compounds. In certain cases, PPO inhibitors such as dithiothreitol (DTT) or metabisulfite were added during the removal of endogenous phenols. However, these inhibitors may irreversibly inactivate PPO.

The aim of this study was to develop a better method for the estimation of total PPO activity from various potato cultivars. Sephadex G-25 chromatography was used to separate endogenous phenolic compounds from the enzyme. In comparison to other methods, the new procedure could efficiently remove phenols and provide high recovery of PPO activity reproducibly. These results demonstrated that the Sephadex G-25 chromatographic method may be used to estimate total PPO activity from potatoes and possibly may be adapted for other plant sources.

### MATERIALS & METHODS

ATLANTIC AND RUSSETT BURBANK potatoes were obtained from Aroostook farm, Presque Isle, ME, and Russett potatoes were obtained from local supermarket. Sephadex G-25 was purchased from Sigma Co., MO. All other chemicals were reagent grade, obtained from commercial sources.

*Authors Hsu, Thomas, and Brauer are with the Eastern Regional Research Center, ARS, U. S. Dept. of Agriculture, 600 East Mermaid Lane, Philadelphia, PA 19118.*

### Crude homogenate preparation

Ten grams peeled potato were diced to small pieces (approx. 2 mm cubes), and then homogenized by a Tissuemizer (Techmarz, Cincinnati, OH) in 20 mL of ice-cold 50 mM sodium phosphate buffer, (pH 6.8) for 5 sec under a stream of N<sub>2</sub> gas. The mixture was cooled for 15 sec on ice and then homogenized for an additional 15 sec. The homogenate was rapidly filtered through four layers of cheesecloth; the filtrate was centrifuged at 8,000 × g for 10 min. The resulting supernatant served as the crude homogenate.

### Polyvinylpyrrolidone (PVPP) absorption method

PVPP (500 mg) (an insoluble, high molecular weight, obtained from Sigma Co.) was mixed with 10 mL of the crude homogenate and then filtered through Buchner funnel. The filtrate was centrifuged at 8,000g for 10 min. The supernatant was referred to as PVPP treated supernatant.

### Ammonium sulfate precipitation method

Ice-cold saturated ammonium sulfate solution was rapidly added to the crude homogenate under constant stirring to a 90% saturation. The mixture was then centrifuged at 40,000g for 30 min, and the pellet was resuspended in 50 mM sodium phosphate (pH 6.8) to a final protein concentration of 1 mg/mL.

### Acetone precipitation method

The acetone precipitation method of Flurkey and Jen (1978) was modified slightly. Five grams finely diced potato in 10 mL acetone, precooled at -40°C, were ground in a mortar with a pestle. The pestle was rinsed with an additional 5 mL iced-cold acetone. The combined acetone extract was centrifuged at 8,000 × g for 10 min at 0-4°C. The pellet was dried overnight under vacuum and then resuspended in 50 mM sodium phosphate buffer, pH 6.8, to a final protein concentration of 1 mg/mL. The resuspended material was referred to as acetone-treated PPO.

### Dialysis method

Dialysis was a combination of methods described in previous reports (Nagahashi, 1985; Hsu et al., 1984). Ten grams diced potato were homogenized in 25 mL of buffer containing 0.2M sucrose, 0.1M Tris, 5 mM sodium metabisulfite and 5 mM β-mercaptoethanol at pH 7.8 with a Techmarz tissuemizer for 30 sec and then centrifuged at 4,000 × g for 10 min. The supernatant was titrated to pH 6.0 with concentrated hydrochloric acid, 500 mg of PVPP was added with stirring. After centrifuging at 8,000g for 10 min, the supernatant was titrated to pH 7.0. PPO was then precipitated by adding saturated ammonium sulfate solution to 90% saturation and collected by centrifugation at 40,000 × g for 30 min. The resulting pellet was resuspended in 50 mM sodium phosphate buffer, pH 7.0 and dialyzed overnight against this buffer.

### Sephadex G-25 chromatography

Two mL of crude homogenate were rapidly applied to a Sephadex G-25 column (1 × 10 cm), and eluted with 50 mM of sodium phosphate buffer, pH 6.8 at 0-4°C (flow rate: 1 mL/min.)

The void fractions of the column were combined and used for determination of PPO activity.

**Assay of polyphenol oxidase**

The procedure previously described by Hsu et al. (1984) was used for PPO assay. In the incubation mixture, proteins obtained by the various methods as described above were allowed to react with 5 mM of dihydroxyphenylalanine (DOPA) in 50 mM sodium phosphate buffer pH 6.8 at room temperature (25°C). The time-dependent absorbance change at 420 nm was measured by a Beckman Model Spectrophotometer.

**Assay of phenols**

Phenols were determined by the modified protocol of Walter and Purcell (1979). An aliquot of the sample was diluted with H<sub>2</sub>O to 1 mL volume. To this, 250 µL of Folin-Dennis (FD) reagent (Swain and Hills, 1959) were added. After 3 min, 500 µL of a saturated solution of sodium bicarbonate were added with agitation. After 1 hr, the solution was centrifuged at 8,000 × g for 10 min, and the absorbance at 725 nm was measured. The phenolic content was determined by using chlorogenic acid as the standard.

**Protein determination**

Protein content was determined by the modified Lowry method (Bensadoan and Weinstein, 1976).

**RESULTS & DISCUSSION**

THE DETERMINATION of PPO activity in crude tissue is often hindered by the presence of endogenous phenolic compounds and their oxidation products. We investigated the possible application of gel-filtration chromatography to rapidly separate the endogenous phenolic compounds from PPO in the crude homogenate obtained from potato tubers. When the crude homogenate of potato extract was chromatographed on a Sephadex G-25 column, two 280 nm positive peaks, one at void volume (Vo) and the other in a later fraction (Ve), were detected (Fig. 1). When aliquots from each fraction of the column were incubated with the PPO substrate, L-DOPA, a single PPO activity peak was observed coincidentally with Vo (Fig. 1). Thus, PPO was recovered in the void volume of the G-25 column. The location of phenolic compounds was determined by incubating aliquots of each fraction with exogenous mushroom PPO, and the 280 nm peak at Vo was found to contain the majority of phenolic compounds. Less than 10% of phe-

nolic compounds were detected in the void volume fraction. These results were confirmed using the Folin-Dennis method (Walter and Purcell, 1979). The vast majority of endogenous phenolic compounds were in the Ve fractions while the Vo fractions were relatively devoided. Thus, G-25 chromatography was an efficient procedure to separate phenols from the PPO. The void fractions showed no browning even after 18 hr at room temperature (25°C). In comparison, the crude homogenate browned rapidly. These results indicated that the PPO in Vo fractions was almost completely free of endogenous phenols.

**Evaluation of the different methods for extracting PPO from potatoes**

The total activity of PPO per grams fresh weight of potato was determined by six different isolation methods (Table 1). Assuming that the activity of crude homogenate to be 100%, PVPP absorption, ammonium sulfate precipitation and column chromatography methods showed quantitative recovery of total PPO activity. The commonly used acetone precipitation and dialysis methods recovered only 60–70% of total PPO activity (Table 1). The lower recovery of PPO activity might be due to inactivation by either acetone or sulfhydryl reagents or incomplete recovery of enzymes during homogenization.

Samples obtained from the above methods were analyzed for phenolic content by Folin-Dennis reagent. The PVPP method alone was not effective in removing phenol since 74% of the phenolic were recovered after treatment. The acetone precipitation and the dialysis method could remove about 80% of the endogenous phenolic compounds. However, gel filtration, the column chromatography, as well as ammonium sulfate precipitation provided an efficient way to remove 85–90% of endogenous phenolic compounds. The enzyme preparation routinely browned during the isolation of PPO by ammonium sulfate procedure which was of concern. In terms of the time for sample processing, both gel-filtration chromatography and the ammonium sulfate method required less than an hour to process one sample. The dialysis method was the most time consuming. The G-25 chromatographic method was best for processing the crude homogenate for the determination of total PPO activity, because the enzyme preparation was low in phenol content, had high PPO activity and was not time consuming.

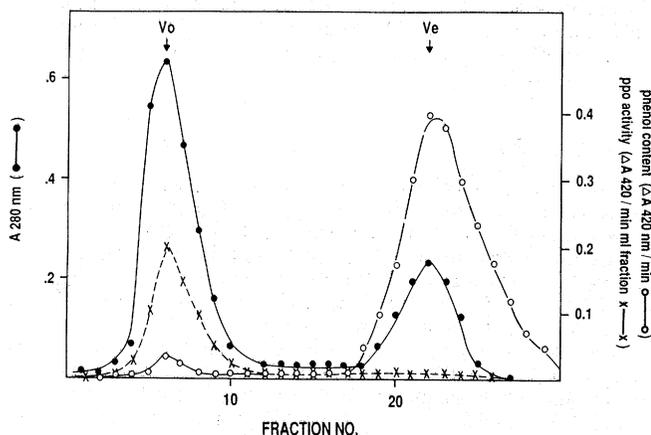


Fig. 1—Sephadex G-25 chromatography of crude homogenate from potato tubers. Crude homogenate of potato tubers was obtained as described under "Materials & Methods." Two mL of crude homogenate were loaded on a Sephadex G-25 column (1 × 10 cm). The column was equilibrated and eluted with 50 mM sodium phosphate, pH 6.8 at 0–4°C. After chromatography, protein was determined by reading absorbance at 280 nm (●—●). An aliquot from each fraction was determined for PPO activity (X—X) and phenol content (O—O) as described under "Materials & Methods."

**PPO activity from various potato cultivars**

To assess the application of the gel filtration method, PPO activity of three different cultivars of potatoes were analyzed. Six samples were prepared from tubers of Russett Burbank, Atlantic and Russett potatoes and assayed for total PPO activity and protein (Table 2). When the data for specific activity were subjected to analysis of variance (Cochran and Cox, 1957), a significant varietal effect was found. The PPO activity of the three cultivars was significantly different from each other at 5% level of probability. The standard deviation within each cultivar averaged less than 15% as estimated from the mean sum of squares of the error term. Therefore, determinations by the gel-filtration method were reproducible and sensitive enough to detect differences in PPO activity among potato cultivars. The protein determination of each cultivar (Table 2) indicated Atlantic potatoes had a higher protein content as compared to other cultivars.

To screen varietal effects and other factors on PPO activity in different cultivars, a rapid and quantitative extraction method is needed. In the development of such a method, caution must be exercised in the selection of extraction procedure. The incomplete recovery of PPO is a common problem in part because PPO can occur in membrane-bound and soluble forms (Ruis, 1972). In this investigation, the crude homogenate was centrifuged before loading onto a G-25 column to remove the debris. Because the vast majority of potato PPO is in soluble

Table 1—Comparison of the different methods for measurement PPO activity of potato samples

	Specific Activity ( $\Delta A$ 420 nm/g F.W.)		Phenol Content <sup>a</sup> ( $\mu\text{g/g}$ F.W.)		Time <sup>b</sup>	No. of Samples Handled
A. Crude	0.92 $\pm$ 0.11 <sup>c</sup>	(100%) <sup>d</sup>	123 $\pm$ 10	(100%)	15 min	1
B. Acetone	0.54 $\pm$ 0.19	(59%)	29 $\pm$ 0.05	(23%)	24 hr	2-6
C. Dialysis	0.64 $\pm$ 0.08	(70%)	21.5 $\pm$ 0.11	(18%)	50 hr	2-6
D. PVPP	0.96 $\pm$ 0.04	(104%)	92 $\pm$ 14	(74%)	25 min	1
E. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.04 $\pm$ 0.11	(112%)	17 $\pm$ 1.0	(14%)	40 min	1
F. Column	0.96 $\pm$ 0.06	(104%)	12 $\pm$ 1.0	(10%)	40 min	1

<sup>a</sup> Phenol content of the samples prepared by each method was determined as described under "Materials and Methods."

<sup>b</sup> Time required to process one sample by each method.

<sup>c</sup> Average and standard deviation from three experiments.

<sup>d</sup> PPO activity from the crude homogenate was assigned 100%. Others were based on the comparison with crude homogenate.

Table 2—Column chromatography method for determination of PPO activity from potato tubers of three cultivars

Potato	Specific activity (units/g F.W.)	Total activity (units)	Protein (mg/g F.W.)
Russett Burbank	1.15 $\pm$ 0.23	4.59 $\pm$ 0.59	3.17 $\pm$ 0.51
Atlantic	0.63 $\pm$ 0.13	2.46 $\pm$ 0.46	5.38 $\pm$ 0.54
Russett	0.83 $\pm$ 0.08	3.22 $\pm$ 0.47	3.18 $\pm$ 0.46

	Analysis of Variance			
	df <sup>b</sup>	SS <sup>c</sup>	MS <sup>d</sup>	F-Test
Total	17	0.96	—	
Variety	2	0.83	0.415	46.1
Error	15	0.13	0.009	

<sup>a</sup> Units were defined as changing the absorbance at 420 nm per minute.

<sup>b</sup> df = degree of freedom

<sup>c</sup> SS = Sum of square

<sup>d</sup> MS = Means of sum of square

form (Ruis, 1972), this centrifugation step resulted in very little loss of PPO activity. If the chromatographic procedure is to be adapted for other plant sources, a lower centrifugation force should be used to avoid the loss of membrane bound PPO. PPO also has been shown to exist in latent forms which can be activated when the homogenate is treated with either detergents or proteases or subjected to freezing and thawing (Angleton and Flurkey, 1984). These forms of PPO, which require the enzyme preparation to be manipulated before the enzyme is active, would not be detected by the current method. At present, this potential source of activity is not of concern because only the catalytically active PPO that would be present after slicing and dicing potato tubers is of interest.

This investigation was aimed at the evaluation of different methods for the recovery of total PPO, not its purification. The quantitation of total PPO activity should be more accurate if only a few steps are involved. As PPO is purified, even by hydrophobic column (Weissman and Lee, 1980), the recovery of active PPO is progressively less. Therefore, hydrophobic column chromatography might be useful for the purification

of PPO but not for determining total PPO activity. Until improved methods are available, the present method utilizing G-25 chromatography should be useful to estimate total PPO activity.

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- Ms received 5/13/88; revised 7/15/88; accepted 7/15/88.

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