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PURIFICATION OF POLYPHENOL OXIDASE FREE OF THE STORAGE PROTEIN PATATIN FROM POTATO TUBER

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Key Word Index—*Solanum tuberosum*; Solanaceae; patatin; polyphenol oxidase; potato tuber; octyl Sepharose.

Abstract—Routine protein purification to homogeneity from potato tuber, as from other storage tissues and seeds, is often hindered due to the large amounts of storage protein present. In potato, patatin, the major storage protein of the tuber, often contaminates preparations. The present work describes the purification of polyphenol oxidase (PPO) from the potato tuber (*Solanum tuberosum* cv Cara) to homogeneity including the critical step of hydrophobic chromatography on Octyl-Sepharose which was sufficient to completely remove patatin. The purified PPO was found to be a doublet of M_r 60 000 and 69 000 when analysed by SDS-PAGE with a K_m 4.3±0.3 mM for L-dihydroxyphenylalanine. Both bands were found to have similar N-termini corresponding to PPO isoforms when sequenced.

INTRODUCTION

Patatin is the major soluble glycoprotein of potato tubers [1] of 40 000 M_r which comprises about 40% of the total soluble protein [2]. Due to this abundance of patatin the purification of other proteins to homogeneity from the potato tuber is made difficult, and preparations are often contaminated with patatin. Furthermore, claims for an M_r of 40 000 for low abundance proteins should be regarded with suspicion as the protein detected may also be the contaminant, patatin.

Polyphenol oxidase; PPO (EC 1.10.3.1) is a copper-containing enzyme which catalyses the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-dihydroxyphenols to *o*-quinones utilizing molecular oxygen [3–5]. These quinones are highly reactive, electrophilic molecules which covalently modify and crosslink to a variety of cellular constituents. The reactions produce undesirable blackening or browning involved in food processing and post-harvest physiology of plant products and is the main reason for the interest in PPO in food technology [6]. It is of particular importance in potato tubers, as it is the enzyme responsible for the discolouration associated with internal blackspot. Although it has been recently subjected to antisense manipulation to lower the occurrence of the blackening response [7], there is still a need for some basic studies at the protein level, particularly the subcellular and tissue localisation of the active level. Any immunological approach to such studies require pure enzyme for the raising of antibody probes. PPO has previously been purified to homogeneity from the trichomes of *Solanum berthaultii* by preparative IEF [8] and the antibodies raised were used to detect levels of a cross-reactive protein in *S. tuberosum* [9].

Although PPO has been partially purified in many plant tissues, reports on its purification to apparent homogeneity are relatively few [8, 10–14] with only one being from potato tuber [15]. The presence of multiple forms, whether due to isoforms or the tanning of proteins during extraction, has made purification of PPO from potato tuber problematic. Although several early attempts were made to characterize the enzyme [6, 16, 17], various molecular weights have been reported. Some of these may have been erroneous due to patatin contamination and degradation of the PPO. The present paper describes the purification of PPO from potato tuber to homogeneity with the major contaminant, patatin, being removed using hydrophobic chromatography on octyl Sepharose.

RESULTS AND DISCUSSION

Polyphenol oxidase has been purified to homogeneity from potato tuber on the criteria of high specific activity and the gel analysis, which gave two bands of M_r 60 000 and 69 000, both having amino acid sequence at the N-terminus identical to translated cDNA sequence for PPO. Figure 1 shows the major protein bands both before and after the final chromatography

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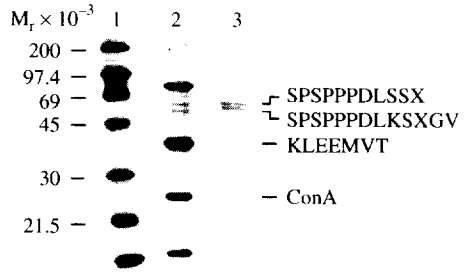

Fig. 1. SDS-PAGE of partially purified and purified potato tuber PPO. Track 1 corresponds to molecular weight markers, track 2 is protein pooled after elution from Con A Sepharose affinity column (35 μg), and track 3 corresponds to the purified PPO doublet obtained after elution on the G-75 column (20 μg). The N-terminal sequences were matched against Blastp data bases. X represents a blank cycle which is probably a cysteine residue by sequence similarities. The sequence of the lower band of PPO is identical to PIR S30930, while the upper band shows 81% similarity but is identical to tomato PPO, S22968. The patatin sequence was identical to Swiss-Prot PAT2$SOLTU.

step on octyl Sepharose with their N-terminal sequences and approximate molecular weights. The lower M_r 40 000 contaminant at the intermediate stage, utilizing lectin chromatography is clearly patatin, illustrating the difficulties encountered by many workers in attempting to purify tuber proteins free of this major storage protein.

We report a purification protocol designed to remove this contaminant which may find wider usage for other enzymes being purified. Track 2 in Fig. 1 corresponds to the fractions pooled following elution from Con A sepharose. Fig. 2 shows the elution pattern after chromatography on octyl Sepharose, and track 3 in Fig. 1 shows the pooled fractions from the G-75 column (profile not shown) which was the purified PPO. A stepwise purification of the enzyme is given in Table 1, the final product showing a 111-fold purification and 25% recovery.

The rationale for the protocol was based on previous purifications of patatin in order to devise a strategy for its removal. Patatin has previously been purified by chromatography on DEAE-Cellulose and ConA Sepharose [1]. ConA affinity chromatography was also used after purification on Bio-gel P-100 and DEAE-Sephacel [18]. However, in the purification protocol reported here, chromatography on ConA alone was not sufficient to remove all of the contaminating patatin. Patatin is a heavily glycosylated protein and exhibits isoforms on SDS gels [2]. These isoforms may be due to alternative glycosylation patterns and this may account for why some of the protein does not bind to ConA. Furthermore, the PPO which does not bind to the ConA possibly reflects lack of glycosylation or sequestration of carbohydrate binding sites due to aggregation.

Patatin exhibits lipid acyl hydrolase [19, 20] activity and it is reasonable to suggest that it would possess some hydrophobicity to allow it to bind its substrates and catalyse the reaction. This characteristic was manipulated in the purification of PPO. Initially chromatography on phenyl Sepharose was attempted but neither patatin or PPO bound to the column. However, Fig. 2 shows the removal of patatin after chromatography on octyl Sepharose where a stronger interaction was achieved and all the patatin was removed. Octyl Sepharose can be used for purification of weakly hydrophobic proteins and it is seen here that patatin binds to the aliphatic octyl group in preference to the aromatic phenyl group of phenyl Sepharose.

As previously reported [21] PPO displayed some hydrophobicity in binding to the column matrix in 1 M $(NH_4)_2SO_4$, but was released from the column in 10 mM phosphate buffer pH 7.0. Hydrophobic interaction chromatography has been used by other authors for the purification of PPO [10–12, 14, 22].

The purified PPO appears as a doublet on the SDS

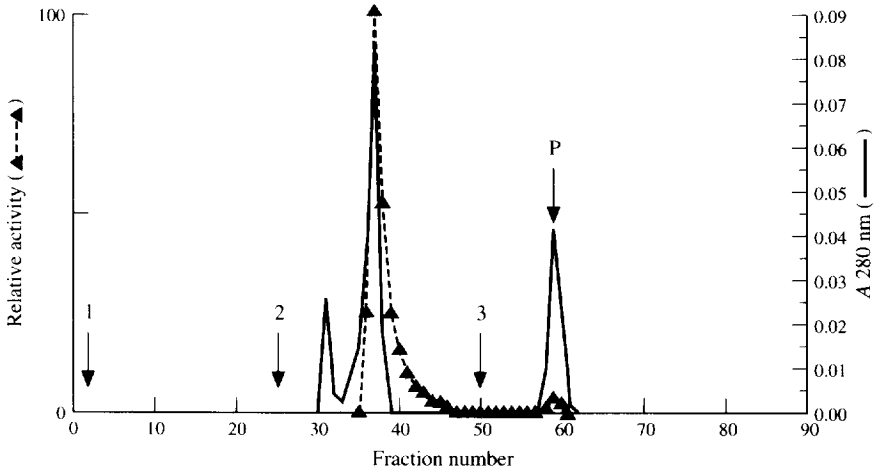

Fig. 2. Removal of patatin (P) from PPO using octyl Sepharose. Buffers used were (1) 10 mM phosphate buffer pH 7.0 containing 1 M $(NH_4)_2SO_4$, (2) 10 mM phosphate buffer pH 7.0, (3) H_2O.

Table 1. Purification protocol for PPO; 1 kg of potato tubers were homogenized and subjected to purification protocol as shown

	Volume (ml)	Protein concn (μg ml^{-1})	Specific activity (ΔAbs 470 nm min mg^{-1})	Total protein (mg)	Total activity (ΔAbs 470 nm min^{-1})	Purification fold	% Recovery
Crude	50	5585	9	279.2	2633	1	100
DEAE	35	4927	12	172.4	2121	1.3	80.4
HR-200	30	1308	49	39.3	1937	5.2	73.5
Con A	70	107	94	7.5	711	10.1	27
Octyl Sepharose	20	37	786	0.877	689	83.3	26.1
G-75	25	25	1046	0.628	657	110.9	24.9

gel with molecular weights of M_r 60 000 and 69 000. Similar molecular weights have been reported previously for PPO from potato tuber [15, 23]. Two PPO cDNAs have been cloned from *S. tuberosum* leaves. Both leaf PPO cDNAs appear to encode polypeptides which are processed to a mature weight of M_r 57 000 [24]. Other authors report molecular weights varying from aggregates of M_r 36 000 monomers [25] to 112 000 [26]. It is thought that these multiple forms and high molecular weights may be artifacts of isolation and purification, generated by quinone alkylation, partial denaturation, or proteolysis [12]. Table 2 shows a comparison of the N-terminal amino acid sequences with those of other PPO proteins. The K_m of the enzyme was 4.3±0.3 mM using L-DOPA as the substrate and assaying spectrophotometrically. The removal of the major protein, patatin, from a crude protein extract of potato tuber by chromatography on octyl Sepharose facilitated the purification of PPO to homogeneity. Use of this matrix may prove useful in the purification of other intractable tuber proteins free of patatin.

EXPERIMENTAL

Materials. Potato tubers cv. Cara were bought locally. All chromatographic matrices were purchased from Sigma, St. Louis except Sephacryl HR-200 and Sephadex G-75 which were obtained from Pharmacia.

Measurement of enzyme activity. Aliquots of samples were added to 900 μl of 10 mM L-DOPA (dihydroxyphenylalanine) in 10 mM phosphate buffer pH 7.0 in a 1-ml cuvette. Initial increase in absorbance was measured with a Phillips PU 8720 spectrophotometer.

Enzyme extraction. The method of [27] was adapted using 1 kg of tubers. Tubers were washed and homogenized with 1 l of cold 200 mM acetate buffer pH 4, 20 mM EDTA, 6% (w/v) Triton X-114. Ascorbic acid (5 mM) was used as an additional measure.

The homogenate was filtered through four layers of cheese cloth and kept at 4° for 90 min to allow pptn of starch grains. This was then centrifuged at 100 000 *g* for 30 min at 4°. The supernatant was collected and the concn of Triton X-114 was increased by an additional 4% (w/v) at 4°. This was then subjected to temperature-induced phase partitioning by warming to 35° for 15 min.

The solution was then centrifuged at 10 000 *g* for 30 min at 25°. The detergent-rich phase was discarded and the clean supernatant containing soluble PPO was brought to 30% satn with solid ammonium sulphate under continuous stirring at 4°. After 1 hr this was centrifuged at 50 000 *g* for 40 min and the pellet was discarded. The ammonium sulphate concn of the supernatant was increased to 60% and again stirred for 1 hr at 4° and centrifuged. This ppt which contained all the PPO activity was re-dissolved in a minimal volume of 10 mM phosphate buffer, pH 7.0, and dialysed against the same buffer. This fr., referred to as crude enzyme, represents the starting material since measurement of enzyme activity and protein was not subject to interference by Triton X-114.

Enzyme purification. Crude enzyme extract following Triton treatment was applied to a DEAE-cellulose column (1.5 × 15 cm) in 10 mM potassium phosphate buffer, pH 7.0. Bound protein was eluted using a gradient of 0–1M NaCl (120 ml) in 10 mM potassium phosphate buffer pH 7.0 at a flow rate of 1 ml min^{-1}. Fractions from the DEAE column with associated PPO activity were pooled and protein pptd with 80% $(NH_4)_2SO_4$. This was resuspended in a minimal volume of 10 mM potassium phosphate buffer pH 7.0 and added to a column of Sephacryl HR-200 (2 × 100 cm) and eluted with the same buffer. Active frs were pooled and dialysed against 20 mM Tris–HCl pH 7.0 containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ (buffer B) and loaded on a Concanavalin A Sepharose (ConA) column (1 × 10 cm) equilibrated

Table 2. N-terminal amino acid sequences of PPO proteins. An alignment of the sequences of the PPO proteins from various tissues was made and absolutely conserved residues are shown in bold. Unknown residues are represented by X

S. tuberosum tuber M_r 69 000	S	**P**	S	**P**	**P**	**P**	**D**	**L**	S	**S**	X				
S. tuberosum tuber M_r 60 000	S	**P**	S	**P**	**P**	**P**	**D**	**L**	K	**S**	X	G	V		
S. tuberosum tuber [23]	A	**P**	A	**P**	**P**	**P**	**D**	**L**	S	S					
S. tuberosum leaf [24]	S	**P**	I	**P**	**P**	**P**	**D**	**L**	K	**S**	C	G	V	A	H
S. berthaulii trichome [30]	S	**P**	I	**P**	**P**	**P**	**D**	**L**	K	S	X	G	V	A	H

in the same buffer. PPO activity which did not bind to the Con A column was brought to 80% $(NH_4)_2SO_4$ and the pellet was resuspended in 10 mM potassium phosphate buffer pH 7.0 containing 1M $(NH_4)_2SO_4$. This was loaded onto an octyl Sepharose column (2 × 20 cm) which had been equilibrated with the same buffer. The column was washed until all unbound protein had been eluted and then washed with 10 mM Potassium phosphate buffer pH 7.0 to elute PPO activity. The patatin, which remained bound was removed from the column by washing with H_2O. The protein with associated PPO activity was then lyophilized and desalted on a G-75 column (2 × 50 cm) in buffer A. All chromatography was carried out at 4°.

Electrophoresis. SDS-PAGE was performed according to the method of Laemmli [28]. The gels used to visualize the frs during the purification process were 12% 1.5 cm minigels and run at 35 mA for approximately 1 hr. Proteins were visualized using Coomassie Brilliant Blue R-250 staining.

Protein sequencing. Protein samples were run on 10% SDS gels prepared with ultra-pure reagents and then electroblotted onto Problott PVDF membrane according to the method of [29]. The protein bands of interest were excised and analysed with an Applied Biosystems 477A protein sequencer.

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