A Modified Method To Purify Patatin from Potato Tubers

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Patatin is a major potato tuber storage protein of approximately 40 kDa that exhibits esterase activity. A purification protocol was developed which involved the following: precipitation of the crude enzyme from potato extracts in stepwise extraction with 40–70% ammonium sulfate followed by chromatography of the most active fractions on Bio-Gel P-100 and of salt gradient fractions on DEAE-Sephacel and Con A–Sepharose. *p*-Nitrophenyl acetate (PNP acetate) and PNP laurate were used to determine the specific activity after each purification step. Enzymatic rates were linear during the assays and were much higher when PNP laurate was used as a substrate. Assay results revealed a 21-fold increase in specific activity of patatin from the crude extract compared to the extract from the Con A column. Preparative SDS–PAGE of the final extract revealed the low molecular weight bands had been eliminated from the Con A fraction.

The protein found in potato tubers is of excellent nutritional quality and is superior to the protein of major cereals and legumes (Kaldy, 1972). One reason is that potato protein is high in lysine, an amino acid in low quantity in most plant proteins (Zwartz et al., 1979). Also, the digestibility of potato protein as measured by PER (protein efficiency ratio) is exceptionally high and is among the best in biological quality among proteins of major crops (Desborough et al., 1981). Of the total soluble potato tuber protein, an estimated 30–40% is patatin, the trivial name given to a family of glycoproteins of approximately 40 kDa as determined by SDS–polyacrylamide electrophoresis (SDS–PAGE) (Racusen et al., 1984; Park et al., 1983). Because the protein is present in such high amounts in potato tubers, it is believed to serve as a storage protein. As a major component of potato tuber protein, the study of patatin and its contribution to nutritional quality is important.

When patatin is analyzed on 12.5% SDS–PAGE, most of the protein is resolved into a broad band composed of overlapping bands of approximately 40 kDa. This broad band indicates that there are only small differences in molecular weight among the members of the patatin family of glycoproteins (Park et al., 1983). When purified patatin of approximately 40 kDa was further analyzed by isoelectric focusing, the patatin family of glycoproteins was separated into many isoforms by charge. The pattern of these isoforms fractionated by charge differs sufficiently among cultivars to be of use in cultivar identification (Desborough et al., 1968; Seibles, 1979; Stegemann et al., 1973; Snyder et al., 1977; Zwartz, 1966). The close structural homology of these forms has been shown by NH_2-terminal sequence analysis (Park et al., 1983). Further molecular characterization including extensive sequence analysis of the patatin multigene family shows that the patatin genes expressed in tuber are very homologous (Mignery et al., 1988).

Normally, patatin is found only in tubers, but it has been found in other potato tissues (roots, stems, and leaves) under environmental and hormonal conditions that interfere with the normal tuberization process (Paiva et al., 1983; Racusen, 1983; Hannapel et al., 1985). Most of the patatin isoforms normally found in tubers are coded for by class I genes. Recently, a distinct isoform has been identified in roots that is coded for by class II genes, which are differentiated from class I genes by the presence of a 22 base pair insert in the 5′-untranslated region (Pikaard et al., 1987) and by differences in the 5′-flanking sequences (Mignery et al., 1988). This isoform is also found in low levels in tubers.

Although considered the major storage protein of potato tubers, patatin has been shown to exhibit lipid acyl hydrolase (LAH) and acyl transferase activities (Racusen, 1984; Desborough, 1983; Brogdon and Dickinson, 1983). These activities were thought to be due to other contaminating proteins. However, Andrews et al. (1988) and Rosahl et al. (1987) used molecular techniques to show unequivocally that patatin proteins do have esterase-type activities. Andrews et al. (1988) used patatin protein isolated from a baculovirus system transformed with a patatin cDNA to show that baculovirus patatin exhibited similar activities to patatin purified from tubers. These activities included high LAH activity with phospholipids, monoacylglycerols, and *p*-nitrophenyl esters as substrates and moderate activity with galactolipids. Rosahl et al. (1987) transformed tobacco cells with the patatin gene and reported a new 42-kDa protein that cross-reacted with patatin antibody and exhibited LAH activity not found in untransformed tobacco controls.

Patatin was originally isolated by Galliard (1971). Patatin can be purified by high-performance liquid gel permeation chromatography (HPL-GPC) (Liedl et al., 1987), but the most widely used method of purification was developed by Racusen and Foote (1980), who reported a 3.6-fold increase in LAH specific activity from the crude extract to the concanavalin A–Sepharose (Con A–Sepharose) eluate. Soluble proteins were extracted from tubers by grinding in 25 mM phosphate buffer and chromatographed sequentially through Sephadex G-50, DEAE-cellulose, and Con A–Sepharose columns. The authors reported that patatin was the only protein observed in the final eluant as visualized on SDS–PAGE.

According to the method of Racusen and Foote (1980), contaminating proteins were observed in the Con A eluate after visualization with silver staining on SDS–PAGE. When Kirschner et al. (1986) purified patatin from Grata with the method of Racusen and Foote, they also observed contaminating bands. Although the Racusen and Foote

method may be adequate for some purposes, a very pure protein is desirable for other purposes such as molecular weight determination, antibody preparation, standards for immunoelectrophoresis, and amino acid analysis. This paper describes a modification of the Racusen and Foote (1980) method which yielded a more pure patatin enzyme than previously reported.

MATERIALS AND METHODS

Materials. DEAE-Sephacel and Con A–Sepharose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). PNP laurate, insoluble polyvinylpolypyrrolidone (PVPP, No. P-6755), and methyl α-D-glucoside were obtained from Sigma Chemical Co. (St. Louis, MO). Bio-Gel P-100, Bio-Rad protein assay dye reagent for the Bradford protein assay, and bovine serum albumin (standard II) were purchased from Bio-Rad Laboratories (Richmond, CA).

Plant Materials. Elite foundation seed potatoes (*Solanum tuberosum* L.), cultivar Superior, were obtained from the Wisconsin Foundation Seed Farm (Rhinelander, WI). Tubers were held at 4 °C for about 200 days prior to use.

Enzyme Assays and Protein Determination. All spectrophotometric assays were performed by using a Varian DMS 100 spectrophotometer at 25 °C. The assay used for LAH activity was that of Racusen (1984) conducted at a wavelength of 410 nm, except that the concentration of the substrate, PNP laurate, was 416.7 μM in 1% (w/v) Triton X-100 and 0.017% (w/v) SDS. During the assays, enzyme rates were found to be linear over time. The kinetics of patatin on this substrate showed that at 416.7 μM the reaction was near V_{max} but not at the 145.2 μM concentration used previously (Racusen, 1984). One unit of activity is defined as 1 μmol of *p*-nitrophenol produced/min. The specific activity was defined as units per milligram of protein. Protein was quantified according to the method of Bradford (1976), conducted at 595 nm, with bovine serum albumin as a standard.

Purification. Potato tubers were washed in mild detergent, rinsed in distilled water, and peeled. Tubers were cut into small pieces and frozen at −20 °C, and 125 g of frozen tuber was ground to a powder in a stainless steel blender with liquid N_2. The powder was homogenized in cold 25 mM phosphate buffer (pH 7.0) with 2 mM sodium bisulfite and 0.1 g of insoluble PVPP per gram of tuber (Andrews et al., 1988; Racusen and Foote, 1980). The homogenate was pressed through three layers of cheesecloth and two layers of Miracloth and centrifuged twice at 10000*g* for 40 min to remove solids.

Salt Fractionation. An aliquot of the supernatant was fractionated from 10 to 80% of saturation in 10% increments with ammonium sulfate. The fractions between 40 and 70% saturated ammonium sulfate contained the highest amount of patatin on the basis of SDS–PAGE and enzyme activity. The remaining supernatant fraction was adjusted to 40% saturation of ammonium sulfate and then centrifuged at 10000*g* for 45 min. The pellet was discarded, and the supernatant fraction was adjusted to a saturation of 70% ammonium sulfate. After centrifugation, the pellet was recovered, resuspended in 30 mL of 25 mM phosphate buffer (pH 7.0) (buffer I), and dialyzed against 18 L of the same buffer for 24 h at 5 °C to remove the ammonium sulfate. The dialysate was frozen to −70 °C and lyophilized.

Molecular Weight Chromatography. All chromatography was performed at 4 °C. The freeze-dried pellet was resuspended in 12 mL of phosphate buffer I with 1 mL of glycerin and loaded onto a Bio-Gel P-100 (7 × 80 cm) column equilibrated with buffer I. Fractions (7.5 mL) were collected and assayed for PNP laurate activity and total protein and electrophoresed on 12% SDS–PAGE. The fractions containing the highest enzyme activity and a high concentration of 40-kDa protein were pooled.

Anion-Exchange Chromatography. The pooled Bio-Gel fractions were loaded onto a (2.5 × 50 cm) DEAE-Sephacel column. The column was equilibrated with 5 bed volumes of buffer I, loaded with sample, and washed with the same buffer until a zero baseline was obtained. The column was eluted with a linear gradient of NaCl (0–0.75 M NaCl in buffer I). The fractions with the highest LAH activity were collected and visualized on 12% SDS–PAGE. Those fractions observed to be the most enriched for the 40-kDa protein and with the fewest contaminating bands on 12% SDS–PAGE were combined and dialyzed overnight against buffer I.

Table I. Purification of Patatin from Potato Tuber

	total act. μmol min^{-1}	% recovered	sp act.,[a] μmol min^{-1} mg^{-1}	increase in act.
total crude extract	1166.5		0.154	
NH_4SO_4 (40–70%)	982.8	84.3	0.200	1.3
MW (Bio-Gel P-100)	345.0	29.6	0.676	4.4
DEAE-Sephacel	268.0	23.0	2.200	14.3
Con A-Sepharose	201.6	17.3	3.300	21.4

[a] Specific activity determined by using 416 μM PNP laurate as substrate.

Affinity Chromatography. This dialysate was lyophilized and resuspended in 25 mM phosphate buffer (pH 7) with 0.5 M NaCl (buffer II). The suspension was applied to a concanavalin A–Sepharose column (2.5 × 20 cm) equilibrated with buffer II and washed with this buffer until a baseline was established. The column was eluted with a linear gradient of 0–80 mM methyl α-D-glucoside in buffer II. Fractions with the highest level of LAH activity and highest concentration of the 40-kDa protein were pooled.

Electrophoresis. SDS–PAGE was performed according to the method of Laemmli (1970). The gels used to visualize the fractions during the purification process were 12% 1.5-cm slab polyacrylamide gels. These gels were run at 60 mA at 25 °C for approximately 6 h. The final gel showing the resultant fractions from each step was a 12% SDS–PAGE minigel (10.0 × 7.0 cm) run at 35 mA at 25 °C for approximately 45 min. Proteins were visualized by using Coomassie Brilliant Blue R-250 and silver staining.

Preparative Gel Electrophoresis. Since no fractions were free from traces of contaminating bands, the pooled fractions highest in LAH activity and judged to have the highest concentration of patatin on SDS–PAGE were further purified by preparative gel electrophoresis. The pooled fractions were dialyzed in 5 mM phosphate buffer (pH 7.0), freeze-dried, and resuspended in 0.025 M Tris buffer (pH 6.8) with 0.192 M glycine and 0.1% SDS (stacking gel buffer) (Laemmli, 1970). The suspension was loaded onto a 12% Laemmli SDS–PAGE with a single slot in the stacking gel and electrophoresed at 45 mA for about 6 h at approximately 25 °C. Gels were stained, and the major band was removed. This band was cut into pieces, placed in dialysis tubing with a molecular weight cutoff of 12 000–14 000, and electrophoresed with buffer II at 45 mV for approximately 24 h until all of the blue stain was removed from the gel pieces. The solution purity was determined on 12% SDS–PAGE. The eluant showed no activity for the PNP laurate substrate. This activity was probably destroyed by the SDS treatment.

Kinetic Studies. Kinetic constants were determined for Con A purified eluant by using PNP laurate as the substrate. Substrate concentrations ranged from 13 to 667 μM. Kinetic constants were calculated by using the kinetic analysis software of the Varian DMS 100 UV–vis dual-beam spectrophotometer.

RESULTS AND DISCUSSION

Eighty-four percent of the LAH activity of potato tubers was recovered in the 40–70% ammonium sulfate fraction (Table I). This step increased the specific activity 1.3-fold. The ammonium sulfate treatment was effective in separating patatin from other proteins (Figure 1). The protein fraction treated with ammonium sulfate (lane 3) showed a higher percentage of protein at 40 kDa with fewer contaminants as compared to the total soluble protein from the crude extract (lane 2).

LAH activity eluted in a single peak (Figure 2a) from the Bio-Gel P-100 column, which coincided with the major peak of protein. This corresponds to the major patatin band at approximately 40 kDa on SDS–PAGE (Figure 1). At least three other small peaks of protein with less than

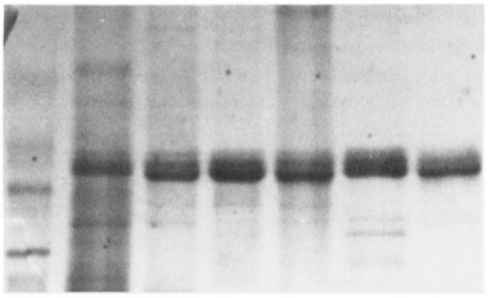

Figure 1. Sequential extracts of protein purification on 12% SDS–PAGE, visualized with silver stain. (Lane 1) Molecular weight markers; (lane 2) total crude extract; (lane 3) 40–70% ammonium sulfate cut; (lane 4) extract from molecular weight column; (lane 5) extract from DEAE-Sephacel column; (lane 6) extract from Con A–Sepharose column; (lane 7) protein purified from preparative PAGE.

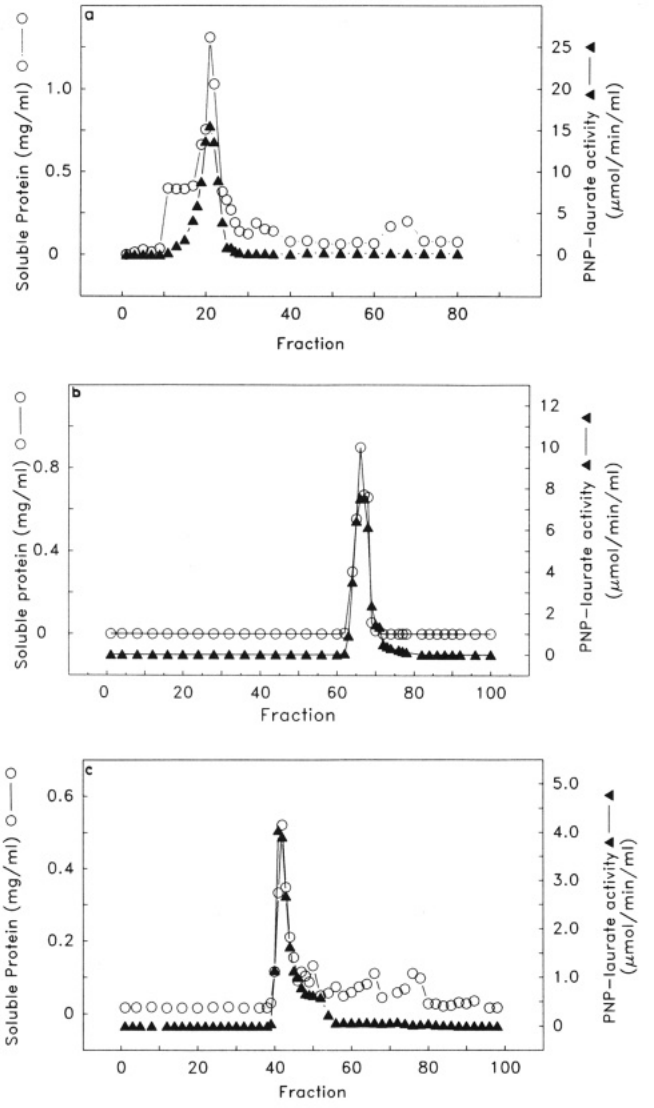

Figure 2. (a) Chromatography of 40–70% ammonium sulfate fraction on Bio-Gel P-100. (b) Anion-exchange chromatography on DEAE-Sephacel of the pooled fractions from the Bio-Gel P-100 column. (c) Chromatography of the pooled DEAE-Sephacel fractions using Con A–Sepharose.

0.02 μM min^{-1} mL^{-1} of LAH activity, which eluted separately from patatin, were observed (Figure 2a). When the fractions highest for LAH activity were visualized on SDS–PAGE, they were found to be further enriched for the 40-kDa protein. (Figure 1, lane 4). Passing the eluant through the Bio-Gel P-100 column resulted in a drop of total activity from 84.3 to 29.6% of the total. The increase in specific activity was 4.4 times that of the crude extract (Table I).

The fractions containing the highest LAH activity and enriched for the 40-kDa protein with the fewest contaminants (as visualized by SDS–PAGE) were pooled and passed through a DEAE anion-exchange column (Figure 1, lane 5, and Figure 2b). The LAH activity eluted as a single peak at approximately 0.50 M NaCl (Figure 2b), which corresponds to the major protein peak. These fractions were enriched for the 40-kDa protein; however, SDS gel electrophoresis still revealed the presence of contaminating proteins (Figure 1, lane 5). For this step, the increase in specific activity was 14.3-fold over that of the crude preparation (Table I).

After dialysis, the fractions containing LAH activity and enriched for the 40-kDa protein (visualized by SDS–PAGE) were pooled and gradient eluted through a Con A column. This partially purified enzyme eluted as a single peak at approximately 33 mM methyl α-D-glucoside (Figure 2c). Other proteins were separated by this procedure, notably three peaks observed between fractions 50 and 80. After this step, the increase in specific activity was 21.4-fold (Table I).

Since contaminating bands were still observed in the pooled Con A–Sepharose fractions (Figure 1, lane 6), these fractions were further purified by preparative SDS–PAGE. No information could be obtained on the effect of this treatment on the increase in specific activity, since SDS destroys all LAH activity. However, a highly purified preparation of patatin was obtained (Figure 1, lane 7).

The kinetics of LAH activity for purified patatin were determined for the substrate PNP laurate. The K_m value was 110.0 ± 8.2 μM.

The five-step purification procedure described herein resulted in a greater increase in specific activity compared to the original method developed by Racusen and Foote (1980). Whereas they obtained a 3.6-fold increase in LAH specific activity over crude extract, this technique yielded a 21-fold increase in specific activity. This increase was similar to that reported by Hasson et al. (1976), who found a 25-fold increase in specific activity during patatin purification. These differences in specific activity may be due to the following reasons. In contrast to Racusen, the first purification step used by both Hasson et al. and this technique was ammonium sulfate precipitation. Another difference was that the specific activity for the crude extract, 2.5 μmol min^{-1} (mg of protein)$^{-1}$, reported by Racusen (1984) differed from the 0.15 μmol min^{-1} (mg of protein)$^{-1}$ reported here (Table I) and the 0.173 μmol min^{-1} (mg of protein)$^{-1}$ [10.4 μmol h^{-1} (mg of protein)$^{-1}$] reported by Hasson et al. (1976). Activity levels were calculated for this method by using the extinction coefficient 17.424 compared to the value of 0.230 used by Racusen. Racusen also used a lower substrate level—145.2 μM compared to the 416.7 μM substrate that was determined to bring the reaction to V_{max}. Although all tested potato cultivars and species have patatin (Racusen et al., 1980; Park et al., 1983), the total amount of patatin as well as esterase activity with specific substrates has been shown to differ among cultivars by Racusen (1986). This method uses the cultivar Superior, whereas Racusen used Kennebec and Desiree. He found that the final activity for PNP laurate for purified patatin from Desiree was much less (0.022) compared to that of Kennebec (3.3). Using his purification method, Racusen reports two different values for Con A purified patatin from Kennebec—3.3 (1983) and 9.1 μmol min^{-1} (mg of protein)$^{-1}$ (1986).

Because patatin has been estimated to be 30–40% of the total soluble tuber protein, the maximum purification can only be 3–4 times that of the crude extract. The 21-fold increase in specific activity throughout purification could be due to an increase in particular isoform(s) of patatin. This may be in part related to the use of ammonium sulfate precipitation also used by Hasson et al. (1976), who reported a 25-fold increase in specific activity during purification. Andrews et al. (1988) found that the patatin isoform from one isolated gene had higher LAH specific activity than total patatin (with isoforms) from tuber. Alternatively, the method may have an effect on enzyme activity, such as removal of an inhibitor or change in protein conformation.

In addition, Racusen and Foote (1980) reported that the Con A eluate contained only a single band at 44 kDa, which they stained with Coomassie Brilliant Blue. However, when the Con A eluant was visualized on SDS–PAGE, contaminating bands were clearly visualized with silver staining, which is much more sensitive than staining with Coomassie Blue (Oakley et al., 1980). When silver staining is used, it is clear that the SDS purification did eliminate some bands that Racusen had not removed with Con A treatment (Figure 1).

The ammonium sulfate treatment was effective in eliminating many contaminating proteins (Figure 1). Although the gel filtration resulted in increased specific activity, it also decreased total activity, perhaps due to selective loss of other proteins with LAH activity. The gradient elution with 0–0.75 M NaCl resulted in a LAH peak at 0.5 M NaCl, which is the single concentration that Racusen and Foote (1980) used. In contrast, the peak for LAH activity for the Con A–Sepharose treatment was at about 33 mM methyl α-D-glucoside, whereas Racusen and Foote used a single concentration of 20 mM. The three protein peaks with no LAH activity that were separated from patatin were not separated when only 20 mM methyl α-D-glucoside was used instead of a gradient (data not shown). Lastly, a shoulder on the peak of LAH activity was observed with the gradient. This may be indicative of some separation of the many patatin isoforms and suggests that a more narrow gradient of methyl α-D-glucoside or the use of another glucoside might be effective in the separation of some of these isoforms.

Thus, the addition of ammonium sulfate fractionation, gradient elution of the DEAE and Con A columns, and preparative SDS–PAGE resulted in a further elimination of contaminants not in the range of 40 kDa (determined by SDS–PAGE) and improved purification. This improved purification should yield a better protein for applications like determination of molecular weight by ultracentrifugation, amino acid profiles, and preparation of antibody for immunoelectrophoresis. When this preparation was run on SDS–PAGE, there appeared to be multiple overlapping bands of approximately the same molecular weight corresponding to the isoforms of patatin (Figure 1). The patatin isolated by this technique would give a better preparation with less contamination for further resolution by IEF or 2-D gels for studies of these isoforms.

The patatin isolated by this technique also would be valuable for studies on the nutritional value of this storage protein. The structures of these isoforms of patatin are sufficiently homologous that these proteins can be validly studied and measured as a group. (Park et al., 1983). Recent evidence by Sonnewald et al. (1989) suggests that the minor differences in mobility of the patatin isoforms (of approximately 40 kDa) on SDS–PAGE are primarily due to the glycan part of the glycoproteins. With deglycosylation of total patatin, the isoform bands differing in mobility are resolved into one band on SDS–PAGE. These minor differences can be ignored if the objective of the researcher is to study patatin on a gross level. Such studies might include the contribution of patatin to overall protein quality in various cultivars and how the protein is affected by treatments during potato tuber growth, storage, and processing.

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