Purification and Characterization of the 22-Kilodalton Potato Tuber Proteins

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ABSTRACT

Three abundant proteins of approximate molecular masses of 22, 23, and 24 kilodaltons were purified from potato (Solanum tuberosum L.) tubers by DEAE cellulose and CM-52 cellulose ion exchange column chromatography, electroelution, and high-pressure liquid chromatography (HPLC). Antibodies specific to the gel-purified 22-kilodalton protein were prepared. Immunoblot analysis showed that the 22-, 23-, and 24-kilodalton proteins are immunologically related and that these proteins are present in tubers and as higher molecular mass forms in leaves, but not in roots, stem, or stolons. The ratios of amino acid composition were compared among the three purified proteins, and the amino-terminal amino acid sequences were determined for these three proteins. All three proteins have identical amino-terminal sequences that match the deduced amino acid sequence of an abundant tuber protein cDNA.

The three major tuber storage protein groups in potato (Solanum tuberosum L.) are the 40-kD glycoprotein, patatin, the 22-kD complex protein group, and the proteinase inhibitors. All three are developmentally regulated in a coordinate fashion during their growth, and accumulation of these three protein families is inhibited by gibberellic acid (5). Patatin, a glycoprotein that constitutes approximately 40% of the soluble protein in potato tubers, was purified by Racusen and Foote (15) by using DEAE cellulose and concanavalin A Sepharose chromatography. Patatin contains about 5% neutral sugar and 1% hexosamine. Isoelectric focusing detected 6 to 10 ionic forms of patatin in the tubers of all cultivars examined. Park et al. (12) reported that the patatin isoforms are heterogeneous within and between varieties. All tuber isoforms are immunologically identical. An immunologically distinct form of patatin also can be detected at much lower levels in the roots (14). Although patatin normally is not detected in stems, leaves, or petioles, it can be induced to accumulate in these organs under certain conditions (11). Proteinase inhibitor II, the most abundant of the proteinase inhibitors present in potato tubers was first isolated by Bryant et al. (4), using Sephadex G-75 gel filtration, and assayed for chymotrypsin and trypsin inhibitory activity. Sanchez-Serrano et al. (17) reported that the proteinase inhibitor II gene is developmentally regulated in potato tubers and environmentally regulated in potato leaves. Upon wounding, the expression of this gene is systemically induced in potato leaves, suggesting that the proteinase inhibitor is involved in the plant defense response against invading predators such as insects or fungi (17). Pena-Cortés et al. (13) have shown that the phytohormone abscisic acid is involved in the wound-induced activation of the proteinase inhibitor II gene in potato and tomato.

Although much research has been devoted to characterizing these two major groups of potato tuber proteins, very little information is available on the 22-kD protein family. Lee et al. (9) identified two cDNA clones, which hybrid selected mRNA that coded for polypeptides with approximate molecular masses of 22 kD. These two cDNAs hybridized to transcripts that were abundant in tubers but were not detected in mRNA from stems or leaves. Stiekema et al. (18) also reported the identification of a tuber-specific cDNA representing a 26.5-kD tuber protein. The developmental regulation of the lower molecular mass tuber proteins indicates that they may play an important role in tuber physiology. Our objective in this study was to extend our knowledge of these proteins in an attempt to identify their function in tuber development. To accomplish this, we have purified these three tuber proteins ranging in molecular mass from 22 to 24 kD and have characterized them by means of immunological and protein chemical methods.

MATERIALS AND METHODS

Plant Materials

Potatoes (Solanum tuberosum L. cv 'Superior') were obtained from the Wisconsin Seed Potato Certification Center, Antigo, WI. Potato plants were grown from tubers under an 8-h photoperiod in the greenhouse under standard conditions. When the plants were harvested, tuber samples were frozen in liquid nitrogen and stored at −70°C.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed in a 0.75-mm 12.5% acrylamide-bis gel by the method of Laemmli (8). Proteins separated on SDS-PAGE were either stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes by using
an electroblotting apparatus for immunoblotting. Immuno-
blots were performed using 22-kD tuber protein antibody
from rabbit and goat anti-rabbit IgG-horseradish peroxidase
conjugate (19). The blots were developed in 0.05% 4-chloro-
1-naphthol in 20 mL of ice-cold methanol plus 0.015% per-
oxide in 20 mM tris and 500 mM NaCl (pH 7.5). Protein
was measured by the dye-binding method of Bradford (3)
standardized with bovine serum albumin.

**Protein Purification**

All purification procedures were carried out at 4°C. Total
tuber protein was extracted by homogenizing 100 g of peeled
tubers in 50 mL grinding buffer with 0.1 g polyvinylpolypyr-
rolidone/g tuber. The homogenization buffer contained 0.2%
diethyl dithiocarbamate and 0.2% sodium bisulfite in 25 mM
sodium phosphate monobasic (pH 7.0). Crude homogenates
were centrifuged at 20,000g for 20 min, and the pellets were
discarded. This preparation was loaded onto a 4 × 30 cm
Sephadex G-50 column (Pharmacia) equilibrated and eluted
with 25 mM sodium phosphate monobasic (pH 7.0). Samples
from the G-50 fraction were then loaded onto a 2.5 × 15 cm
DEAE (diethyl aminoethyl)-cellulose column (Whatman),
which had been equilibrated with 25 mM sodium phosphate
monobasic (pH 6.5). Proteins were partially purified by elution with a linear
salt gradient 0 to 500 mM NaCl in 10 mM sodium phosphate
monobasic (pH 6.5). DEAE effluent was collected, concen-
trated, and desalted using a Centricon microconcentrator
(Amicon) and then loaded onto a 1.5 × 12 cm CM (carboxy
methyl)-52 cation exchange column (Whatman) that had been
equilibrated with 10 mM sodium phosphate monobasic (pH
6.5). Gradient conditions were 0 to 100 mM NaCl in 10 mM sodium phosphate
(pH 6.5) over 40 min and absorbance was read at 280 nm.

**Electrophoretic Elution**

Partially purified CM-52 fractions were combined, desalted,
and loaded onto a 12.5% preparative SDS-PAGE with the
addition of sodium thioglycolate (0.1 mM) to the cathode
buffer reservoir (6). After staining with Coomassie brilliant
blue R-250 for 15 min and destaining once with 7% acetic
acid and three times with deionized water for 5 min each, the
protein bands of interest were sliced away from the rest of
the gel. The excised gel band was then diced into approximately
1.0-mm² pieces, and the protein was electroeluted by the
method of Bhown et al. (2) with an electrophoretic sample
concentrator (ISCO, model 1750).

**Preparation of Specific Antisera**

Antisera were raised in New Zealand white rabbits against
the gel-purified 22-kD tuber protein. After 7 to 10 mL of

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**Table I. Purification of the 22-kD Potato Tuber Protein**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein</th>
<th>Percent 22-kD Protein Present in Total Protein Fraction*</th>
<th>22-kD Protein Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-50 Sephadex effluent</td>
<td>247 mg</td>
<td>10%</td>
<td>24.7 mg</td>
<td>100%</td>
</tr>
<tr>
<td>CM-52 cellulose effluent</td>
<td>52.5 mg</td>
<td>62%</td>
<td>12.1 mg</td>
<td>49%</td>
</tr>
<tr>
<td>HPLC, S-300</td>
<td>9.4 mg</td>
<td>100%</td>
<td>9.4 mg</td>
<td>38%</td>
</tr>
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</table>

* Total tuber protein was extracted from 100 g of tuber tissue (cv 'Superior').

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Figure 1. SDS-PAGE of tuber proteins at different stages of purifi-
cation. Total tuber proteins (lane 1), DEAE anion exchange effluent
(lane 2), CM-52 cation exchange eluent (lane 3), electroeluted 22-kD
protein (lane 4), and standard molecular mass markers (MW).
preimmune serum had been collected, immunization was initiated by subcutaneous injection of the 22-kD protein mixed in Freund's complete adjuvant. Booster injections were administered after 14, 21, and 28 d in Freund's incomplete adjuvant (7). The amount of protein injected varied from 0.5 to 0.8 mg/injection. Antiserum batches were collected after 38 d by heart puncture and stored at −20°C.

Amino Acid Analysis

Determination of the ratios of the amino acid composition was performed as described previously (10). After separation of the purified tuber proteins by SDS-PAGE, they were electrophoresed onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) in CAPS buffer (10 mM 3-[cyclohexylamino]propanesulfonic acid and 10% methanol [pH 11.0]) and submitted to the Iowa State University Protein Facility for amino acid analysis and amino-terminal sequencing. Amino acid analysis was performed with the Applied Biosystems 420A derivatizer, 130A separation system and a 920A data analysis system. The analysis of purified lysozyme was included as a control. Amino-terminal sequence analysis was performed on an Applied Biosystems 477 protein sequencer as described previously (10). DNA sequence analysis was performed using standard procedures as reported by Stiekema et al. (18).

Figure 2. HPLC profiles of the 23- and 24-kD tuber proteins (A) and the 22-kD tuber protein (B). Zero elution volume refers to the start of the sample injection. The dotted lines represent an isocratic gradient (A), 100 mM NaCl in 10 mM sodium phosphate monobasic (pH 6.5), and a linear gradient (B), 0 to 100 mM NaCl in 10 mM sodium phosphate monobasic (pH 6.5). Proteins of Peak 3 (A) were resolved on the linear gradient (B).

Figure 3. SDS-PAGE and immunoblot analysis for HPLC-purified 22-, 23-, and 24-kD tuber proteins. Total protein extracts from tubers (lanes 1 and 4), pure 22-kD protein (lanes 2 and 5), and pure 23-, 24-kD protein (lanes 3, 6) were resolved by SDS-PAGE. Two identical gels were used for a Coomassie blue staining (lane 4–6) and immunoblot analysis (lanes 1–3) using an antibody specific for the purified 22-kD tuber protein. Standard molecular mass markers (MW) are also shown.
RESULTS

Purification of the 22-kD Potato Tuber Proteins

The protocol followed in the purification of the 22-kD potato tuber protein is summarized in Table I and Figure 1. When G-50 Sephadex total potato tuber proteins (Fig. 1, lane 1) were subjected to chromatography on DEAE-cellulose in 25 mM sodium phosphate monobasic, most of the higher molecular mass proteins including patatin were adsorbed to the column. The fraction that did not bind to this anion exchange column (DEAE effluent) contained most of the lower molecular mass tuber proteins, including the 22-kD complex (see arrows, Fig. 1, lane 2). The DEAE effluent was loaded onto a CM-52 cation exchange column and partial purification was obtained using a linear (0–500 mM) salt gradient (Fig. 1, lane 3). After the partially purified 22-kD protein was collected, essentially pure 22-kD protein was obtained using electroelution and resolved as a single band on SDS-PAGE (Fig. 1, lane 4). However, the 23- and 24-kD proteins could not be purified by this gel electroelution procedure. DEAE effluent was loaded onto a SYNCHROPAK S300 (HPLC) and yielded a single peak (Fig. 2A, arrow, 23,24-kD) on an isocratic salt gradient (Fig. 2A, dotted line). This peak resolved two bands with approximate molecular mass of 23 and 24 kD on SDS-PAGE (Fig. 3, lane 6). The peak containing the 22-kD protein (Fig. 2A, peak 3) was collected, reloaded onto a SYNCHROPAK S-300 (HPLC) linear salt gradient 0 to 100 mM NaCl (Fig. 2B, dotted line), and produced two peaks (Fig. 2B). The second peak (Fig. 2B, arrow) resolved as a single band with molecular mass of 22 kD (Fig. 3, lane 5). This 22-kD fraction represented a 38% yield and a 10-fold purification (Table I).

Immunoblot Analysis

Antibody specific to the gel-purified 22-kD protein reacted with three bands of approximately 22, 23, and 24 kD in total tuber protein (Fig. 3, lane 1), whereas the 22-kD antibody reacted to only one band in HPLC purified 22-kD protein fraction (Fig. 3, lane 2), and two bands in the HPLC purified 23- and 24-kD proteins (Fig. 3, lane 3). Despite their differences in size, these three proteins (22, 23, and 24 kD) are immunologically related as shown by immunoblotting with antibody specific for the 22-kD protein.

To examine the distribution of the 22-kD tuber protein in different parts of the potato plant, immunoblots were performed with protein extracts from leaf, stem, root, tuber, petiole, stolon, and new tubers (Fig. 4). Immunoblot analysis shows that this protein was present in mature tubers, newly initiated tubers, 28-d petioles which had been induced to accumulate the tuber proteins (Fig. 4, lanes T, NT, and 28).

Table II. Ratios of the Amino Acid Composition of Four Major Potato Tuber Proteins

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>22 kD</th>
<th>23 kD</th>
<th>24 kD</th>
<th>Patatin*</th>
<th>Lysozyme</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Calculated</td>
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<tr>
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<td>3</td>
<td>3</td>
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<tr>
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<td>3</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>6</td>
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<tr>
<td>Leu</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>9</td>
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<tr>
<td>Lys</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>3</td>
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<tr>
<td>Met</td>
<td>&lt;1</td>
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<td>&lt;1</td>
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<tr>
<td>Val</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

* Racusen and Foote (15). * Ratios were calculated by setting histidine composition in each protein to equal one. Cysteine and tryptophan were not determined.

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and as a higher molecular mass form in leaves (Fig. 4, lane L). The positive reaction with protein from swollen stolons (designated as new tubers) shows that the 22-kD tuber protein accumulates early in tuber development similar to patatin’s pattern of accumulation (11). The 22-kD protein antibody does not react with protein from stems, roots, petioles from whole plants, or stolons from nontuberizing plants (Fig. 4).

Amino Acid Analysis

A comparison of the ratios of the amino acid composition of these three proteins (Table II) shows that they are essentially equivalent. Despite their differences in size, 6 of the 15 amino acid ratios are identical, and only one (valine) differs by more than one unit. The amino acid composition of patatin (16), by contrast, shows a number of significant differences.

To examine more closely the relationship of these three purified proteins, the amino-terminal sequence of each protein was determined. Despite charge and molecular mass differences Figure 5 shows that the amino-terminal sequence of all three proteins is identical. The first 21 amino acids of the purified 22-kD protein are identical to the deduced amino acid sequence of the potato cDNA clone, p34021, starting with Leu-41 through Ile-61 (Fig. 6). This potato cDNA represents an abundant tuber mRNA with the capacity to code for a protein of approximately 26.5-kD molecular mass. The nucleotide sequence of this tuber cDNA was first reported by Stekemra et al. (18). Discrepancies between the amino acid sequence of the purified 22-kD protein and the deduced amino acid sequence of p34021 prompted us to reevaluate the nucleotide sequence of p34021. The corrected portion of the amino acid sequence of p34021 (from codon 57 to 85) is shown in Figure 6 with changes in the nucleotide sequence underlined.

**DISCUSSION**

One approach to the developmental study of the process of tuberization has been the characterization of the major tuber proteins. To date, however, the controlling factors involved in this growth process are not clear. Recent advances in understanding potato tuber development have been made at the molecular level studying the major tuber proteins, patatin, and proteinase inhibitor II. Both have known metabolic activities, and their functional roles in the potato plant have been discussed (15, 17), but much less information is available on the 22-kD tuber proteins (ranging in molecular masses from 20 to 25 kD). The 22-kD proteins have been shown to be present in the tubers of all cultivars so far examined (DJ Hannapel, unpublished results). They are also present in

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 kD</td>
<td>Lou - Val - Leu - Pro - Glu - Val</td>
</tr>
<tr>
<td>23 kD</td>
<td>Lou - Val - Leu - Pro - Glu - Val - Tyr - Asp - Gin - Asp - Gly - Aen - Pro</td>
</tr>
<tr>
<td>24 kD</td>
<td>Lou - Val - Leu - Pro - Glu - Val - Tyr - Asp - Gin - Asp - Gly - Aen - Pro</td>
</tr>
</tbody>
</table>

**Figure 5.** A comparison of the amino acid sequence of the amino-termini of the 22-kD potato tuber proteins.

Protein from tubers formed in vitro and can be induced to accumulate along with patatin in petioles from a petiole-leaf cutting system (11). These proteins accumulate very rapidly in tubers during early development but are detected only in very low levels, if at all, in other parts of the plant. Because of their complexity and charge variance, however, the 22-kD tuber proteins have not previously been purified. By comparison, the purification of patatin and proteinase inhibitor II by classical purification methods has been relatively straightforward (4, 16).

To broaden our knowledge of the 22-kD protein complex, we have purified three proteins with approximate molecular masses of 22, 23, and 24 kD by ion exchange column chromatography and electrophoresis. The electrophoresed 22-kD protein was shown to be pure by SDS-PAGE and immunoblot analysis. The 22-kD protein eluted as one peak on the chromatogram from the HPLC cation exchanger and resolved as one band on SDS-PAGE. However, the 23- and 24-kD proteins could not be separated from one another using HPLC. A single band of either 23 or 24 kD could be electrophoresed, but analysis of the isolated band showed that a clear separation was not obtained. Purification could not be improved by repeating the electrophoresion of either the 23- or 24-kD proteins. These two proteins eluted as two peaks on the chromatogram from the HPLC cation exchanger, and each peak was shown to resolve as two bands with molecular masses of 23 and 24 kD on SDS-PAGE. The paired proteins from both sets are immunologically cross-reactive with antibody specific to the 22-kD tuber protein (data not shown). Aside from an artificial explanation, the simplest interpretation of these results is that the 23- and 24-kD protein complex represents two related proteins of different molecular mass but with the same charge. Separate and distinct aggregations of these two proteins could produce charge differences resulting in the resolution of the twin peaks on the chromatogram.

Despite molecular mass and charge differences among these three proteins, they are immunologically related and the ratios
of their amino acid composition are essentially equivalent. Amino acid sequence analysis showed that the amino-terminal sequence of all three proteins is identical. Twenty-one amino-terminal amino acids of the purified 22-kD tuber protein matched the deduced amino acid sequence of the tuber cDNA, p34021, which has open reading frames to code for a 26.5-kD protein (18). The different molecular mass forms of the 22-kD family could be products of different genes from this small multigene family (18), but the amino-terminal sequence data suggests that the three tuber forms could be products of the same gene which are modified posttranslationally. The amino-terminus of all three tuber proteins represents a cleavage site 40 amino acids downstream from the first codon of p34021. The hydrophobic nature of this sequence indicates that it is a signal peptide associated with membrane attachment (18). Using immunoblot analysis, a higher molecular mass form (approximately 27-kD) was also detected in leaves. Northern blot hybridization using p34021 as a probe, showed transcripts present in the leaves of some field-grown plants early in the growing season (DJ Hannapel, S-G Suh, unpublished data). It is likely that the high molecular mass leaf form is the product of a unique gene from the p34021 multigene family which is differentially expressed. Alternatively, the leaf form could be a product of the p34021 gene with a different pattern of posttranslational modification comparable to the different forms of the large subunit of ribulose bisphosphate carboxylase in maize (1).

Stiekema et al. (18) found that the deduced amino acid sequence of p34021 has 50% homology to the carboxy-terminal 90 amino acids of the Kunitz trypsin inhibitor of winged bean. The presence of the hydrophobic signal sequence is in agreement with the expected cellular location of proteinase inhibitors in vacuoles (20). Preliminary studies on our part have shown that some of the purified 22-kD proteins are potent inhibitors of trypsin and chymotrypsin. Future work will focus on conclusively proving the proteinase inhibitor activity of these proteins and demonstrating the wound-inducibility of their genes.

LITERATURE CITED