Purification and characterization of the 22-kilodalton potato proteinase inhibitors

Sang-Gon Suh
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Purification and characterization of the 22-kilodalton potato proteinase inhibitors

Suh, Sang-Gon, Ph.D.
Iowa State University, 1990
Purification and characterization of the 22-kilodalton potato proteinase inhibitors

by

Sang-Gon Suh

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1990
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Tuberization in potato is a complex process leading to the differentiation of an underground stem, stolon, into a storage organ, the tuber (Artschwager, 1924). During this developmental process, morphological and genetical changes such as radial expansion of the stolon take place and the expression of specific genes is influenced. This is reflected by the synthesis of starch and specific proteins (Lee et al., 1983; Park et al., 1983). This differentiation process can be influenced by environmental conditions such as photoperiod, temperature, or nutrition (Gregory, 1956; Hammes and Nel, 1975; Menzel, 1980; Ewing and Wareing, 1981). Plant hormones also play an important role in tuber development. Cytokinin enhances tuber formation whereas gibberellic acid inhibits tuber development. (Forsline and Langille, 1976; Hannapel, et al., 1985; Menzel, 1980). Both physiological and hormonal factors controlling tuber formation have been studied. However the molecular mechanisms of tuberization are unknown.

Since the advent of gel electrophoresis and chromatography, a more complex picture of potato proteins has been revealed. SDS-polyacrylamide gel electrophoresis of proteins from potato tubers reveals a simple pattern which differs drastically among organs of the plant (Paiva et al., 1983). The most prominent protein band, which can make up to 40% of the total soluble protein, has a molecular weight of about 40 kD and corresponds to a group of glycoproteins purified by Racusen and Foote (1980) using ion-exchange and affinity chromatography and given the trivial name patatin.
Due to its high accumulation in tubers, patatin is considered as a storage protein. Patatin is generally detected in tubers, but can be induced to accumulate elsewhere (Paiva et al., 1983). Although the exact physiological roles of patatin remain unknown, the patatin is generally considered to have esterase activity and is termed a lipid acyl hydrolase (Racusen, 1984). This enzymatic activity of patatin might function in protection against microbial growth (Racusen, 1984). Patatin exhibits extensive charge heterogeneity when analyzed by nondenaturing polyacrylamide gel electrophoresis or isoelectric focusing (Park et al., 1983). The expression of the patatin is under strict developmental control. Using cDNAs encoding patatin as a probe, it was demonstrated that under normal conditions, patatin mRNA can not be detected in leaves, stems, and roots of tuber-forming potato plants (Mignery et al., 1984). Recently, the isolation and analysis of patatin gene has been accomplished (Pikaard et al., 1987; Rosahl et al., 1986). Pikaard et al. (1987) showed that two classes of patatin genes exist. Class I is exclusively expressed in tubers while Class II patatin genes are expressed in both tubers and roots. Wenzler et al. (1989) reported that patatin promoter had sucrose-inducible expression in transgenic plants.

Proteinase inhibitors, the other major proteins of potato, also have been extensively studied. The proteinase inhibitors that are wound-inducible in potato and tomato leaves are among a group of several proteins originally discovered in potato tubers, where they appear early during tuberization and continue to accumulate until the tubers are mature (Ryan et al. 1976). The two inhibitors can account for over 10% of the total
soluble proteins of potato tubers, depending upon the variety (Ryan et al., 1976). Proteinase inhibitor I was purified by using affinity chromatography, has a native molecular weight of 41000 and is composed of subunits with molecular weights of 8100 (Plunkett et al., 1982). Proteinase inhibitor II, the most abundant of the proteinase inhibitors present in potato tubers, was first isolated by Bryant et al. (1976) using Sephadex G-75 gel filtration, has a dimeric molecular weight of 21000, is composed of subunits with molecular weight of 10500, assayed trypsin and chymotrypsin inhibitory activity. The proteinase inhibitor I and II from potato display chymotrypsin and trypsin/chymotrypsin inhibitory activity, respectively (Bryant et al., 1976; Garcia-Olmedo et al., 1987; Plunkett et al., 1982; Ryan, 1984). Sanchez-Serrano et al. (1986) reported that the proteinase inhibitor II gene is developmentally regulated in tubers and environmentally regulated in potato leaves. Upon wounding, the expression of this gene is systemically induced in nonwounded potato leaves (Johnson and Ryan, 1990; Keil et al., 1989; Sanchez-Serrano et al., 1986; Thornburg et al., 1987). The proteinase inhibitor II gene also was induced by abscisic acid (Pena-Cortes et al., 1989). The induction of this gene by cytokinin (BAP) and ethylene (ethophon), and its inhibition by auxin were demonstrated by Kernan and Thornburg (1989).

All three proteins (patatin, 22-kD proteins, and proteinase inhibitors) are developmentally regulated in a coordinated fashion during tuber growth (Paiva et al., 1983; Hannapel, unpublished results, Department of Horticulture, Iowa State University). Although much research has been devoted to characterizing the patatin and the proteinase inhibitors, very
little information is available on the 22-kD tuber protein family. To extend our knowledge of the potato tuber proteins, we report the purification of several tuber proteins ranging in molecular weights from 22 to 24 kD. My objectives in this study were to focus on the purification of the 22-kD potato tuber proteins and the identification of their cellular function and elucidation of their gene expression. To this end, I purified three tuber proteins ranging in molecular weight of 22 to 24 kD by using several protein-chemical methods, and characterized these three proteins by immunoblotting and amino acid sequence analysis. I revealed that the 22-kD proteins were potent inhibitors of trypsin and/or chymotrypsin. The genes coding for the 22-kD proteins are developmentally regulated in tubers and environmentally expressed in leaves in response to mechanical wounding.

Explanation of Dissertation Format

This dissertation is arranged in the alternate format consisting of two papers, one of which is accepted in Plant Physiology and the other will be submitted to a scientific journal. Sang-Gon Suh was the principal investigator on all research reported herein, and is the first author on both papers. Drs. Hannapel and Hall served as co-major Professors for Sang-Gon in his research.
SECTION I. PURIFICATION AND CHARACTERIZATION OF THE 22-KILODALTON POTATO TUBER PROTEINS
PURIFICATION AND CHARACTERIZATION OF THE 22-KILODALTON POTATO TUBER PROTEINS

Sang-Gon Suh, Jon E. Peterson, Willem J. Stiekema

and David J. Hannapel
ABSTRACT

Three abundant proteins of approximate molecular weights of 22, 23, and 24 kilodalton (kD) 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-kD protein were prepared. Immunoblot analysis showed that the 22-, 23-, and 24-kD proteins were immunologically related and that these proteins were present in tubers and as higher molecular weight forms in leaves, but were not detectable in stems, roots, and 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.
INTRODUCTION

The three major tuber storage protein groups in potato (*Solanum tuberosum* L.) are the 40-kilodalton (kD) glycoprotein, patatin, the 22-kD complex protein group, and the proteinase inhibitors. All three are developmentally regulated in a coordinated fashion during tuber growth, and accumulation of these three protein families is inhibited by gibberellic acid (Hannapel et al., 1985). Patatin, a family of glycoproteins that constitutes approximately 40% of the soluble protein in potato tubers, was purified by Racusen and Foote (1980) by using DEAE (diethyl aminoethyl) 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. (1983) 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 (Pikaard et al., 1987). Although patatin normally is not detected in stems, leaves, or petioles, it can be induced to accumulate in these organs under certain conditions (Paiva et al., 1983). Proteinase inhibitor II, the most abundant of the proteinase inhibitors present in potato tubers was first isolated by Bryant et al. (1976), using Sephadex G-75 gel filtration, and assayed for chymotrypsin and trypsin inhibitory activity. Sanchez-Serrano et al. (1986) reported that the proteinase inhibitor II gene is developmentally regulated in noninduced 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 (Sanchez-Serrano et al., 1986). Pena-Cortes et al. (1989) 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. (1983) identified two cDNA clones, which hybridized to mRNAs that encoded for polypeptides with approximate molecular weights of 22 kD. These two cDNAs hybridized to transcripts that were abundant in tubers but did not hybridize to transcripts obtained from stems or leaves. Stiekema et al. (1988) also reported the identification of a tuber-specific cDNA representing a 26.5-kD tuber protein. The developmental regulation of the lower molecular weight 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 three tuber proteins ranging in molecular weights 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 polyacrylamide gel electrophoresis was performed in a 0.75-mm 12.5% acrylamide-bis gel by the method of Laemmli (1970). 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. Immunoblots were performed by using 22-kD tuber protein antibody from rabbit and goat anti-rabbit IgG-horseradish peroxidase conjugate (Towbin et al., 1979). The blots were developed in 0.05% 4-chloro-1-naphthol in 20 ml of ice-cold methanol plus 0.015% hydrogen peroxide in 20 mM tris and 500 mM NaCl (pH 7.5). Protein was measured by the dye-binding method of Bradford (1976) standardized with bovine serum albumin.

Protein Purification

All purification procedures were carried out at 4°C. Total tuber protein was extracted by homogenizing 100 grams of peeled tubers in 50 ml
grinding buffer with 0.1 g polyvinylpolypyrrolidone/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 20,000 g for 20 min, and the pellets were discarded. This preparation was loaded onto a 4 x 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 x 15 cm DEAE (diethyl aminoethyl)-cellulose column (Whatman), which had been equilibrated with 25 mM sodium phosphate monobasic (pH 7.0). DEAE effluent was collected, concentrated, and desalted using a Centricon microconcentrator (Amicon) and then loaded onto a 1.5 x 12 cm CM (carboxy methyl)-52 cation exchange column (Whatman) that had been equilibrated with 10 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 also applied to a 4.6 x 250 mm SYNCHROPAK S300 strong cation exchange column with a high-pressure liquid chromatography (HPLC, Waters), 721 programmable system and M730 Data Module system. One-hundred-microgram samples of protein were injected per run with a flow rate of 1.0 ml per min. 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 (Hunkapiller et al., 1983). 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 and Bennett (1983) 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 7-10 ml of 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 (Hurn and Chantler, 1980). 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 (Matsudaia, 1987). After separation of the purified tuber proteins by SDS-PAGE, they were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) in CAPS buffer (10 mM 3-[cyclohexylamino]-1-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 (Matsudaia, 1987). DNA sequence analysis was performed using standard procedures as reported by Stiekema et al. (1988).
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 1 and Fig. 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 weight 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 weight 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 to 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 weights 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 weight of 22
kD (Fig. 3, lane 5). This 22-kD fraction represented a 38% yield and a tenfold purification (Table 1).

**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 tissues, 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, Lane T, NT, and 28) and as a higher molecular weight 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 (Paiva et al., 1983). The 22-kD protein antibody does not react with protein from stems, roots, petioles from whole plants, or stolons from nontuberizing plants (Fig. 4).
A comparison of the ratios of the amino acid composition of these three proteins (Table 2) 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 (Racusen and Foote, 1980), 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 weight differences, Fig. 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 weight. The nucleotide sequence of this tuber cDNA was first reported by Stiekema et al. (1988). 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 Fig. 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 (Racusen, 1984; Sanchez-Serrano et al., 1986), but much less information is available on the 22-kD tuber proteins (ranging in molecular weights from 20 to 25 kD). The 22-kD proteins have been shown to be present in the tubers of all cultivars so far examined (Hannapel, unpublished results, Department of Horticulture, Iowa State University) data not shown). They are also present in protein from tubers formed in vitro and can be induced to accumulate along with patatin in petioles from a petiole-leaf cutting system (Paiva et al., 1983). 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 (Bryant et al., 1976; Racusen and Foote, 1980).

To broaden our knowledge of the 22-kD protein complex, we have purified three proteins with approximate molecular weights of 22, 23, and
24 kD by ion exchange column chromatography and electroelution. The 
electroeluted 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 electroeluted, but 
analysis of the isolated band showed that a clear separation was not 
obtained. Purification could not be improved by repeating the 
electroelution 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 weights 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 artifactual explanation, the 
simplest interpretation of these results is that the 23- and 24-kD protein 
complex represents two related proteins of different molecular weight 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 weight 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 (Stiekema et al., 1988). The different molecular weight forms of the 22-kD family could be products of different genes from this small multigene family (Stiekema et al., 1988), but the amino-terminal sequence data suggests that the three tuber forms could be products of the same gene which are modified post-translationally. 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 (Stiekema et al., 1988). Using immunoblot analysis, a higher molecular weight 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 (Hannapel and Suh, preliminary results, Department of Horticulture, Iowa State University). It is likely that the high molecular weight 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 post-translational modification comparable to the different forms of the large subunit of ribulose bisphosphate carboxylase in maize (Bedbrook et al., 1979).

Stiekema et al. (1988) found that the deduced amino acid sequence of p34021 has 50% homology to the carboxyl-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 (Walker-Simmons and Ryan, 1977).
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 investigating the proteinase inhibitor activity of these proteins and demonstrating the wound-inducibility of their genes.
LITERATURE CITED


Fig. 1. SDS-PAGE of tuber proteins at different stages of purification. 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 weight markers (MW)
Fig. 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)
Fig. 3. SDS-PAGE and immunoblot analysis for HPLC-purified 22-, 23-, and 24-kD tuber proteins. Total protein extracts from tubers (lane 1, 4), pure 22-kD protein (lane 2, 5) and pure 23-, 24-kD protein (lane 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 weight markers (MW) are also shown.
Fig. 4. Immunoblot analysis of the 22-kD tuber protein in different organs of potato. The immunoblot was performed using an antibody specific for the purified 22-kD tuber protein with total protein extracts from mature tubers (T), stems (S), leaves (L), roots (R), petioles taken directly from whole plants (0), petioles induced to accumulate tuber proteins for 28-d (28), nontuberizing stolons (ST), and newly initiated tubers (NT). Prestained standard molecular markers (MW) are also shown
Fig. 5. A comparison of the amino acid sequence of the amino-termini of the 22-kD potato tuber proteins

24 kD  Leu - Val - Leu - Pro - Glu - Val

23 kD  Leu - Val - Leu - Pro - Glu - Val - Tyr - Asp - Gln - Asp - Gly - Asn - Pro

22 kD  Leu - Val - Leu - Pro - Glu - Val - Tyr - Asp - Gln - Asp - Gly - Asn - Pro
Fig. 6. A comparison of the first twenty-one amino-terminal amino acids of the purified 22-kD tuber protein with the deduced amino acid sequence (residues 41 through 61) of a 26.5-kD tuber protein. The deduced amino acid sequence was derived from the corrected sequence of the tuber cDNA clone, p34021 (Stiekema et al., 1988). Corrections were made by the addition of a guanine at codon 57 and the deletion of an adenine at codon 85.
Table 1. Purification of the 22-kilodalton potato tuber protein

<table>
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<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Percent 22-kD protein present in total protein fraction</th>
<th>22-kD protein (mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tr>
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<td>2. DEAE cellulose effluent</td>
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<td>3a. CM-52 cellulose</td>
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<td>3b. HPLC, S-300 cation exchanger</td>
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<td>9.4</td>
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<td>10</td>
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*The total tuber protein was extracted from 100 g of tuber tissue (cv. 'Superior').

*Percent 22-kD protein present in each fraction was estimated by scanning with a densitometer the Coomassie brilliant blue stained bands of the SDS-PAGE profiles (shown in Figure 1) for each protein fraction.
Table 2. Ratios of the amino acid composition of four major potato tuber proteins

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<th>amino acid</th>
<th>22 kD</th>
<th>23 kD</th>
<th>24 kD</th>
<th>patatin**</th>
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*Ratios were calculated by setting histidine composition in each protein to equal one. Cysteine and tryptophan were not determined.

**Racusen and Foote (1980).
SECTION II. PROTEINASE INHIBITOR ACTIVITY AND WOUND-INDUCIBLE GENE EXPRESSION OF THE 22-KILODALTON POTATO TUBER PROTEINS
PROTEINASE INHIBITOR ACTIVITY AND WOUND-INDUCIBLE
GENE EXPRESSION OF THE 22-KILODALTON
POTATO TUBER PROTEINS

Sang-Gon Suh, and David J. Hannapel
ABSTRACT

Using a proteinase inhibitor gel determination assay, we have demonstrated that the 22-kilodalton (kD) potato tuber proteins were potent inhibitors of serine proteinases. Wound induction of the genes coding for the 22-kD potato tuber proteins also was detected at the RNA level. Two out of three purified proteins from the 22-kD family of potato tuber protein were effective inhibitors of both trypsin and chymotrypsin. The third purified protein with molecular weight of approximately 24 kD inhibited only trypsin activity. Comparison of the amino acid sequence of the putative reactive sites of several members of the proteinase inhibitors with the deduced sequence of the 22-kD protein revealed that the 22-kD protein contained sequences which could potentially possess "double-headed" sites of inhibition, one against trypsin and the other against chymotrypsin. Like other potato proteinase inhibitors, the genes coding for the 22-kD proteins were developmentally regulated in tubers and environmentally regulated in leaves. In leaves, transcripts of the 22-kD protein family were detected 6 h after wounding and were highest after 12 h in locally wounded leaves. The strongest induction occurred systemically in nonwounded leaves in response to mechanical wounding. Cross-hybridization of a cDNA, p34021, which codes for the 22-kD tuber protein, with proteinase inhibitor I, and II cDNAs and a second family of 20-kD potato tuber cDNAs revealed no cross-homology. Members of this second group of 20-kD potato tuber proteins also exhibited wound-induction in leaves at the RNA level. This study identifies an entirely new group of potato proteinase inhibitors whose regulated expression is similar to
proteinase inhibitors I and II.
A number of plants respond to wounding by insects or other mechanical damage within several hours by accumulating proteinase inhibitors in wounded and nonwounded leaves (Ryan, 1984). The defense reaction of the plant against wounding or insect invasion is either restricted to a defined region near the original wound (local reaction) or may also occur in tissue far from the original wound site (systemic reaction) (Pena-Cortes et al., 1988). In potato plants, wounding induces two small multi-gene families that code for two inhibitors of serine proteinases called proteinase inhibitor I (monomer Mr 8100) and proteinase inhibitor II (monomer Mr 12300) (Ryan, 1984). Members of both gene families are developmentally regulated during the process of tuberization, and environmentally regulated in leaves in response to chewing insects or other mechanical wounding (Ryan, 1984; Thornburg et al., 1987). Proteinase inhibitor II mRNA from potato plants can be detected locally in wounded leaves and systemically in nonwounded leaves (Pena-Cortes et al., 1988; Sanchez-Serrano et al., 1987). Johnson and Ryan (1990) have shown that high levels of sucrose can activate the proteinase inhibitor II promoter in transgenic plants while others have implicated the hormones auxin and abscisic acid as controls in the regulation of potato proteinase inhibitor gene expression (Kernan and Thornburg, 1989; Pena-Cortes et al., 1989).

The kinetics of proteinase inhibitor RNA accumulation has been studied extensively (Graham et al., 1986). Proteinase inhibitor II RNA can be induced to accumulated in leaves as early as 20 min in response to wounding.
or after treatment with the oligosaccharides, chitosan (Pena-Cortes et al., 1988). Tomato proteinase inhibitors I and II mRNA can be induced to accumulate in wounded leaves 4 to 6 h following wounding, reaching maximal levels about 10 h after wounding, and then declining rapidly up to 20 h after wounding (Graham et al., 1986). The tomato inhibitor I gene also is regulated in a wound-inducible manner in transgenic nightshade plants (Solanum nigrum) (Johnson et al., 1990). Systemic wound-inducible expression of the potato proteinase inhibitor II gene in stems and leaves of transgenic potato and tobacco plants also was reported (Keil et al., 1989). In poplar trees, transcripts hybridizing to a cDNA clone (win 3) with significant homology to Kunitz trypsin inhibitors systemically accumulated in the unwounded leaves of wounded plants (Bradshaw et al., 1989).

In previous work, we reported the purification and characterization of the 22-kD potato tuber proteins (Suh et al., 1990). Very little information is available about these developmentally-regulated proteins, and their function is unknown. Despite molecular weight and charge differences among these three proteins, the amino-terminal sequences of the 22-, 23-, and 24-kD proteins were identical and the amino acid composition of the three proteins was equivalent (Suh et al., 1990). By comparing the amino acid sequence of the purified 22-kD protein to the deduced sequence from a cDNA clone, we were able to match the purified protein to the tuber cDNA, p34021 (Stiekema et al., 1988). This cDNA had significant homology to the amino acid sequence of a Kunitz trypsin inhibitor from winged bean (Stiekema et al., 1988). In this report, we demonstrate the proteinase inhibitor activities of the purified 22-, 23-, and 24-kD tuber proteins and
wound-inducible expression in leaves, both locally and systemically. Like the other major tuber storage proteins, the 22-kD family of proteins was also developmentally regulated in tubers. This study identified an entirely new class of potato proteinase inhibitors.
MATERIALS AND METHODS

Plant Materials

Potato plants (Solanum tuberosum L. cv. Superior) were grown under standard greenhouse conditions for the wounding experiments. Potato leaves were wounded according to Pena-Cortes et al. (1988); the fourth, fifth and sixth leaves from the apex of 40 to 50 cm tall plants were wounded in a 0.5 cm-wide block between the mid-vein, 3 to 4 blocks per leaf with a hemostat. Leaves were harvested 0, 6, 12 and 24 h after wounding. In the text, the following nomenclature will be used: upper or lower leaf refers to positions relative to the wounded leaf. For developing tuber studies, plants were grown under an 8 h photoperiod in the greenhouse for 2 weeks. Petiole-leaf cuttings were taken from these induced plants (grown under a shortday photoperiod) and grown in moist perlite-vermiculite (3:1 by volume) for 21 d. As tubers developed from the axillary buds of these cuttings (Paiva et al., 1982), they were excised, frozen in liquid nitrogen and stored at -80°C. Axillary tubers were harvested at 0, 1, 2, 4, 6, 8, 10, 14, and 21 d after cuttings were taken. Zero-day buds were axillary buds taken directly from whole plants.

Proteinase Inhibitor Gel Determination Assay

The procedure for purifying the 22-, 23- and 24-kD proteins was described previously (Suh et al., 1990). The gel determination assay was based on the methods of Laskowski and Sealock (1971). Five micrograms of either patatin (purified according to Racusen and Foote, 1980), bovine
serum albumin (BSA, Sigma B 2518) or phosphorylase b (Sigma P 6635) was incubated with 0.1 μg of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma T 8462) or TLCK (1-chloro-3-tosylamide-7-amino-1-2-heptanone)-treated chymotrypsin (Sigma C 3142) and various inhibitors in 100 mM sodium borate (pH 8.0), and 5 mM calcium chloride. Equivalent amounts (1.0 μg) of the purified 22-, 23-, or 24-kD proteins were added to the reaction buffer before incubation at 37°C for 6 h. Trypsin/chymotrypsin digestions were stopped by adding 1/2 volume of sodium dodecyl sulfate (SDS) extraction buffer (30 mM tris-HCl, 1.5% SDS, 2.5% β-mercaptoethanol) to the reaction buffer and boiling for 2 min. The samples were then run on 0.75 mm, 12.5% SDS-polyacrylamide gels by the method of Laemmli (1970), and stained with Coomassie brilliant blue R-250. Proteins were measured by the dye-binding method of Bradford (1976) standardized with bovine serum albumin.

Filter Hybridization

Total RNA was extracted from developing axillary tubers and wounded leaf tissue by the phenol/chloroform method of Dix and Rawson (1983). Polyadenylated RNA was isolated from total RNA by chromatography on oligo (dT) cellulose (Collaborative Research, Grade T-3) as described by Mignery et al. (1984). For northern blots, RNA was subjected to electrophoresis in 1.4% agarose gels containing 5 mM methyl mercury hydroxide (Thomas, 1980) in a tris borate buffer and blotted onto a nylon filter. Slot-blot hybridization also was performed on nylon filter according to the method of Thomas (1980). Filters were hybridized to a 32P-labeled, nick-translated
gel-purified insert from the cDNA clone, p34021 or from the 20-kD cDNA p749 as previously described (Mignery et al., 1984).
RESULTS

Inhibition of Trypsin and Chymotrypsin Activity by
the 22-kD Tuber Proteins

Stiekema et al. (1988) reported that the deduced amino acid sequence of p340621 has 50% homology to the carboxyl-terminal 90 amino acids of the Kunitz trypsin inhibitor of winged bean. To determine whether the purified 22-kD protein has proteinase inhibitor activity, we used a modified proteinase inhibition gel determination assay (see materials and methods). We first examined the inhibitory effects of the purified 22-kD tuber protein on tryptic digestion using three protein substrates (Fig. 1). The three protein substrates, patatin (Fig. 1, lanes 1, 2, 3, and 4), bovine serum albumin (lanes 5, 6, 7, and 8), and phosphorylase b (lanes 9, 10, 11, and 12) were incubated with trypsin (lanes 2, 6, and 10) in 100 mM sodium borate (pH 8.0), and 5 mM calcium chloride buffer for 6 h. As expected, no tryptic digestion occurred in the negative control reactions (protein substrate only, lanes 1, 5, and 9) while complete or partial tryptic digestion occurred in the positive control reactions (protein substrate plus trypsin, lanes 2, 6, and 10). The protein substrates treated with trypsin plus Bowman-Birk trypsin/chymotrypsin inhibitor (Birk, 1985) produced protected, intact protein bands (lanes 3, 7, and 11). The proteinase inhibitor tightly binds to the trypsin active site and trypsin can not digest the substrate (Laskowski and Sealock, 1971). Protein substrates treated with trypsin plus the purified 22-kD tuber protein also resulted in protected, intact protein bands (lanes 4, 8, and 12). When
equivalent amounts (usually 1.0 ug) of Bowman-Birk inhibitor or purified 22-kD protein were incubated with the substrate and trypsin, we found that the 22-kD tuber protein had as potent an inhibiting effect on trypsin activity as the Bowman-Birk inhibitor of soybean (compare lanes 3 and 4).

To test the 22-kD tuber protein's effect on chymotrypsin activity, we conducted the same gel determination assay as in Fig. 1 using chymotrypsin. As shown in Fig. 2, protein substrates treated with chymotrypsin plus the 22-kD tuber protein also resulted in protected intact protein bands (lanes 4, 8, and 12). Upon evaluation of this trypsin/chymotrypsin assay, we determined that, of the three protein substrates, phosphorylase b was the most sensitive to tryptic and chymotryptic digestion. Therefore, further analysis of trypsin and chymotrypsin digestion was performed using phosphorylase b as a substrate. Our data showed that the 22-kD tuber protein is a potent inhibitor of trypsin and chymotrypsin. To determine whether the 23- and 24-kD tuber proteins also exhibit proteinase inhibitor activity, we conducted proteinase inhibitor gel determination assays with phosphorylase b as a substrate and used potato proteinase inhibitor II and the Bowman-Birk inhibitor as controls (Fig. 3, lanes 6, 12 and 7, 13). The purified 23-kD tuber protein protected the protein substrate from proteolytic digestion by both trypsin and chymotrypsin (Fig. 3, lanes 4 and 10), while the 24-kD tuber protein had an inhibitory effect on trypsin activity but was ineffective in inhibiting chymotrypsin activity (Fig. 3, lanes 5 and 11).
Comparison of the Putative Reactive Sites for Trypsin and Chymotrypsin

The proteinase inhibition gel determination assays have shown that the 22-kD protein is a potent inhibitor of both trypsin and chymotrypsin activity and, therefore, is likely to be a "double-headed" inhibitor similar to potato proteinase inhibitor II (Plunkett et al., 1982). In an attempt to identify the reactive sites for this new proteinase inhibitor, the putative reactive sites derived from the deduced amino sequence of the 22-kD cDNA, p34021 (Stiekema et al., 1988) were compared with the reactive sites of several representative proteinase inhibitors (Fig. 4). The putative reactive sites of the 22-kD protein are a Lys-Leu for trypsin (Fig. 4, P₁ - P₁') and a Leu-Gly for chymotrypsin (Fig. 4, P₁ - P₁'). The leucine residue at position P₁ of the chymotrypsin site is conserved among several of the proteinase inhibitors represented in Fig. 4 (boxed). One disulfide loop (Cys-Cys) was readily distinguished for the chymotrypsin reactive site in the 22-kD protein sequence. This disulfide loop is conserved in both the potato and tomato proteinase inhibitor II sequences shown in Fig. 4 (boxed). However, one cysteine is conserved in the trypsin reactive site (Fig. 4, position P₂', boxed). The only other cysteine residue present in the 22-kD protein is located 50 amino acids upstream (Cys-82, Suh et al., 1990) from the P₁ trypsin position. The conserved amino acid residues of the compared reactive sites are shown boxed in Fig. 4.
Cross-hybridization and Developmental Regulation

Slot-blot hybridization was performed to determine if there was any cross-homology between p34021 and cDNAs representing a second distinct family of 20-kD tuber proteins or proteinase inhibitor I and II cDNAs (Fig. 5). Using p34021 as a probe, no cross-hybridization was detected among any of the other potato cDNAs (Fig. 5). The cDNA clones p749 and p740 have been selected from a tuber-specific cDNA library by hybridization to the 20-kD tuber protein cDNAs pT190 and pT216, respectively (Lee et al., 1983).

Developing axillary tubers (Paiva et al., 1982) from petiole-leaf cuttings of the cv 'Superior' were used to study the developmental regulation of the 22-kD tuber protein in total RNA fractions using northern blot hybridization with p34021 as a probe. Fig. 6 shows that p34021 transcripts (estimated to be about 1000 nucleotides in length) can be detected as early as 6 d after cuttings have been taken, with a steady increase as tubers develop. With longer exposures of hybridized filters, trace amounts of p34021 transcripts can be detected as early as 2 d after cuttings have been taken. Similar temporal patterns of accumulation have been observed in this developmental system for p749, p740, and patatin transcripts (Hannapel, unpublished results). Fig. 6 also shows a second higher molecular weight band detected by labeled p34021 inserts (lanes 10, 14, and 21). This higher molecular weight band was not detected in polysomal (Fig. 6, lane T) or polyadenylated RNA fractions (Fig. 7) and was resistant to degradation by RNAse-free deoxyribonuclease (data not shown).
Wound-Inducible Expression of the 22-kD Tuber Protein Gene

The expression of proteinase inhibitor I and II genes has been reported to be wound-inducible in potato and tomato (Graham et al., 1986; Sanchez-Serrano et al., 1986). To determine if 22-kD protein gene expression can be induced by wounding in potato plants, we studied the effect of mechanical wounding on RNA accumulation in potato leaves. Fig. 7 shows the results from northern blot hybridization of polyadenylated mRNA isolated from nonwounded and wounded leaves of potato plants hybridized with either the $^{32}$P-labeled potato tuber cDNAs p34021 (A) or p749 (B). In order to follow the time course of accumulation of the wound-induced 22-kD protein mRNAs, leaves were harvested at 0, 6, 12 and 24 h after wounding. No transcripts for either cDNA were detected in mRNA from nonwounded control leaves (Fig. 7, lane 0), whereas transcripts were detected 6 h following wounding (Fig. lane 6). The levels of p34021 and p749 transcripts were highest at 12 h (Fig. 7, lane 12), and both types decreased after 12 h with the p34021 transcript level dropping significantly by 24 h (Fig. 7, lane 24). A trace amount of p34021 mRNA was detected in unwounded control leaves (Fig. 7, 12 h, lane C). Both p34021 and p749 transcripts accumulated to their highest levels in nonwounded upper systemic leaves (12 h, lane U). Substantially less mRNA accumulation was observed in lower systemic leaves after wounding (12 h, lane L). This same pattern of induction was also reported for both proteinase inhibitors I and II (Graham et al., 1986). The cDNA clone, p740, which is in the same gene family as p749, also had similar kinetics of induction as p34021 and p749 (data not shown).
DISCUSSION

Our data indicated that all three 22-kD proteins had proteinase inhibitory activity as potent as other known proteinase inhibitors (Garcia-Olmedo et al., 1987). We speculate that the 22-kD protein has putative reactive sites for both trypsin and chymotrypsin based on the deduced amino acid sequence from p34021. Comparison of the 22-kD protein's reactive sites with several other families of known proteinase inhibitors reveals that the reactive sites of 22-kD protein, Bowman-Birk inhibitor of soybean, proteinase inhibitor II from potato and tomato are Lys-Leu, Lys-Ser, Arg-Glu and Arg-Glu against trypsin and Leu-Gly, Leu-Ser, Leu-Asn and Phe-Asn against chymotrypsin, respectively. The $P_1$ positions of the reactive sites are Lys and Arg for trypsin specificity and Leu and Phe for chymotrypsin specificity. The putative reactive sites for the 22-kD protein are consistent with the known mechanistic rules for inhibitor reactive sites (Laskowski and Sealock, 1971). The Bowman-Birk inhibitor possesses two independent sites of inhibition against trypsin and chymotrypsin (Birk, 1985). The putative reactive sites of the 22-kD protein also constitute "double-headed" inhibitory sites for trypsin and chymotrypsin. The 22-kD protein contains four cysteine residues. Three out of four cysteines are conserved with the proteinase inhibitor II from potato and tomato at the putative reactive sites. The peptide bond is encompassed in the disulfide loop (Cys-Cys) which ensures that during the binding of the inhibitor to the proteinase the two peptide chains cannot dissociate (Garcia-Olmedo et al., 1987). Based upon this reactive site comparison, no homology was
observed between the 22-kD protein and the Kunitz trypsin inhibitor at the putative reactive site, although Stiekema et al. (1988) reported 50% homology between the C-terminal amino acid sequences of p34021 and the Kunitz trypsin inhibitor. Several conserved sequences were observed with proteinase inhibitor II, however no cross-homology was detected with slot-blot hybridization. The \( \text{P}_4 \) position of the reactive site is Lys and Leu for trypsin and chymotrypsin, respectively, in both the Bowman-Birk inhibitor and the 22-kD protein, while no sequence homology was observed between these two inhibitors (Birk, 1985). From this perspective, the 22-kD proteins could represent a new class of proteinase inhibitors.

The 22- and 23-kD proteins both have inhibitory activity against trypsin and chymotrypsin, whereas the 24-kD protein has inhibitory activity against trypsin with no specificity for chymotrypsin. Previously we reported (Suh et al., 1990) that although these three proteins have equivalent amino acid composition and identical amino-terminal sequences, they had distinct molecular forms as resolved by SDS-PAGE. It is conceivable that a structural modification producing the 24-kD molecular weight form resulted in the loss of chymotrypsin reactivity. Despite using several different batches of the purified 24-kD protein in several assays, we were not able to show that the 24-kD protein had any specificity for chymotrypsin inhibition. The standard incubation period used in the gel determination assays was 6 h. Similar protection results were obtained with the 22-, 23-, and 24-kD proteins for tryptic digestion and the 22- and 23-kD proteins for chymotryptic digestion after 16 h incubation periods. Chymotryptic digestion of the protein substrate occurred even after 1 h
incubation periods with the 24-kD protein (data not shown). Aside from an
artifactual explanation, we hypothesize that i) these proteins could be the
products of distinct but related genes from the same multi-gene family, or
ii) the three different forms may arise from modification of a common gene
product. In genomic Southern blot analysis, we observed seven bands
ranging in size from 4.3 to 17 kb which hybridized to p34021 in the
tetraploid potato cv, 'Superior' (data not shown). Stiekema et al. (1988)
also reported that p34021 mRNAs are encoded by a small multi-gene family.
Therefore, it is quite plausible that these proteins are the products of
separate genes from the same family.

The amino-terminus of all three 22-kD tuber proteins begins at a
cleavage site 40 amino acids downstream from the first codon of p34021,
which has open reading frames to code for a 26.5 kD protein (Stiekema et
al., 1988; Suh et al., 1990). This 26.5 kD preprotein contains a 5'
hydrophobic signal sequence indicating an association with membrane
attachment, possible post-translational modification in the Golgi complex,
and storage in vacuoles. Packaging in vacuoles would be in agreement with
the expected cellular location of proteinase inhibitors (Walker-Simmons and
Ryan, 1977). Patatin and proteinase inhibitors I and II also are
synthesized with a signal peptide which allows the polypeptide to enter the
lumen of the endoplasmic reticulum (Blobel, 1980). In the Golgi complex,
these proteins may undergo co- or post-translational modification to form
the mature proteins which are sequestered into the vacuoles of tuber or
leaf cells (Sonnewald et al., 1989a; Shumway et al., 1970). The major
potato tuber protein, patatin, was reported to be glycosylated and targeted
into vacuoles of transgenic plants (Sonnewald et al., 1989b). Such a post-translational modification could explain both the size differences and charge heterogeneity observed among the three proteins. An attached side chain could block the chymotrypsin reactive site and alter the biological activity. Further analysis will be necessary to identify the putative attached groups which have modified the function of the 24-kD trypsin inhibitor.

Like potato proteinase inhibitor I and II, the p34021 gene is developmentally regulated in tubers and environmentally regulated in leaves. Northern blot hybridization showed that the 22-kD tuber protein mRNA accumulated very early during tuber development in the axillary bud tuber developmental system (Fig. 6). Our data showed a higher molecular weight band accumulated synchronously with the relatively abundant lower molecular weight p34021 transcripts. Based on preliminary studies, this high molecular weight RNA could be the product of a distinct but related gene from the multi-gene family, or it could represent an unprocessed precursor RNA which is not present in polysomal or polyadenylated RNA fractions. The pattern of 22-kD protein accumulation during early tuber morphology is temporally coordinate with accumulation of proteinase inhibitors I and II, patatin, and the other storage proteins (Paiva et al., 1983; Hannapel, unpublished results, Department of Horticulture, Iowa State University). Differences have been noted, however, between the specific tuber cell types in which, for example, patatin and proteinase inhibitor II accumulated (Keil et al., 1989). Different kinetics of induction, however, are involved in the environmental regulation of proteinase
inhibitor genes in leaves in response to wounding. For example, the accumulation of proteinase inhibitors I and II in potato leaves occurred within 4 to 6 h following wounding and is preceded by the accumulation of their respective mRNAs (Graham et al., 1986). These two genes also are systemically induced in nonwounded leaves and upper stem segments of wounded plants, whereas no induction occurred in nonwounded roots or lower stem segments (Pena-Cortes et al., 1989). Our data shows the 22-kD tuber protein mRNAs are also induced to accumulate in leaves in response to mechanical wounding. Locally wounded leaves accumulated 22-kD protein mRNAs as early as 6 h after wounding and these mRNAs continued to increase up to 12 h. Like potato proteinase inhibitor II, the strongest levels of induction for the 22-kD proteinase inhibitor occurred systemically in nonwounded upper leaves of wounded plants. A trace amount of p34021 mRNA was detected in unwounded control leaves (Fig. 7, 12 h, lane C). This low level of induction could be the result of mechanical damage during watering in the greenhouse. Surprisingly, a second distinct family of 20-kD proteins, represented in this study by the potato cDNA p749, demonstrated a similar pattern of expression to the 22-kD proteinase inhibitor in response to wounding. This second family of 20-kD tuber proteins has not been characterized and its function is unknown. Despite the similarities in kinetics of induction in response to wounding among proteinase inhibitor I and II, the 20-kD protein, and the 22-kD protein, no cross-homology was observed between p34021 (22-kD) and the other, respective, cDNAs. On this basis, it is assumed that p34021 represents a unique new class of potato proteinase inhibitors. Further research efforts will focus on the
isolation of 22-kD genomic clones, the comparison of homologous regulatory sequences among different proteinase inhibitor genes and the identification of tissue-specific and wound-responsive elements.
LITERATURE CITED


Fig. 1. SDS-PAGE of trypsin inhibitor gel determination assay. Three different substrates, patatin (lanes 1-4), BSA (lanes 5-8), or phosphorylase b (lanes 9-12) were incubated with either trypsin alone (lanes 2, 6, 10), trypsin plus Bowman-Birk inhibitor (lanes 3, 7, 11) or trypsin plus the purified 22-kD tuber protein (lanes 4, 8, 12). Protein substrate without inhibitors and trypsin (lanes 1, 5, 9) and standard molecular weight markers (MW) are also shown.
Fig. 2. SDS-PAGE of chymotrypsin inhibitor gel determination assay. Three different substrates, patatin (lanes 1-4), BSA (lanes 5-8), or phosphorylase b (lanes 9-12) were incubated with either chymotrypsin alone (lanes 2, 6, 10), chymotrypsin plus Bowman-Birk inhibitor (lanes 3, 7, 11) or chymotrypsin plus the purified 22-kD tuber protein (lanes 4, 8, 12). Protein substrate without inhibitors and chymotrypsin (lanes 1, 5, 9) and standard molecular weight markers (MW) are also shown.
Fig. 3. SDS-PAGE of trypsin/chymotrypsin inhibitor gel determination assay. Phosphorylase b was used in all lanes as a substrate. Substrate alone and protein substrate plus trypsin or chymotrypsin as a positive control are shown (lanes 1, 2, and 8, respectively). Protein substrate plus trypsin and various inhibitors are shown in lanes 2-7 and substrate plus chymotrypsin and various inhibitors are shown in lanes 8-13. The following test and control inhibitors are used in the reaction buffer: 22-kD protein (lanes 3, 9), 23-kD protein (lanes 4, 10), 24-kD protein (lanes 5, 11), Bowman-Birk inhibitor (lanes 6, 12), and proteinase inhibitor II from potato (lanes 7, 13)
Fig. 4. Alignment of the amino acid sequence of the reactive sites of several members of the proteinase inhibitor families. 22-kD is deduced sequence of p34021 (Stiekema et al., 1988); PI II (P) is proteinase inhibitor II from potato (Thornburg et al., 1987); PI II (T) is inhibitor II from tomato (Graham et al., 1985b); BBI is Bowman-Birk inhibitor of soybean (Birk, 1985); PI I (P) is inhibitor I from potato (Cleveland et al., 1987); PI I (T) is inhibitor I from tomato (Graham et al., 1985a); WBI is Kunitz trypsin inhibitor from winged bean (Yamamoto et al., 1983). P₃-P₃' represents the putative reactive site which interacts with the active site of the enzyme. Vertical arrows (*) indicate the putative reactive bonds. Conserved positions are boxed.
Fig. 5. Slot-blot hybridization of several major tuber protein cDNAs, using p34021 as a probe. The cDNA, p34021, codes for a 22-kD tuber protein (Stiekema et al., 1988). The plasmids p749 and p740 are representative of the 20-kD tuber protein family; pT1 24 and pT2 47 are proteinase inhibitors I and II, respectively (Graham et al., 1985a,b); and pGMO1 represents a patatin cDNA (Mignery et al., 1984). Thirty nanograms of purified p34021 insert was bound to the filter for hybridization. All other samples contained 100 ng of purified insert.
Fig. 6. Northern blot hybridization of total RNA extracted from developing tubers from the axillary buds of a petiole-leaf cutting system (see Materials and methods) showing the accumulation of 22-kD tuber protein mRNA. Ten μg of total RNA was loaded for each sample. Total RNA was extracted from developing tubers harvested at 0 d (lane 0), 1 d (lane 1), 2 d (lane 2), 4 d (lane 4), 6 d (lane 6), 8 d (lane 8), 10 d (lane 10), 14 d (lane 14), and 21 d (lane 21). After blotting, the filter was hybridized to the $^{32}$P-labeled cDNA insert from p34021. Three micrograms of polysomal tuber RNA was loaded in Lane T.
Fig. 7. Northern blot hybridization of polyadenylated mRNA extracted from leaves of wounded potato plants, cv. 'Superior'. Locally wounded leaves from greenhouse-grown plants were harvested 6 h (6), 12 h (12) and 24 h (24) after wounding. Nonwounded upper (U) and lower (L) leaves (relative to locally-wounded leaves) from wounded plants were harvested 12 h after wounding. Control leaves were harvested from nonwounded plants at the start of the experiment (0) or 12 h after initiating the experiment (12, C). Filters were hybridized with $^{32}$P-labeled inserts from either the 22-kD tuber cDNA, p34021 (A) or the 20-kD tuber protein cDNA, p749 (B). Five micrograms of polyadenylated mRNA was loaded for each sample. Three micrograms of the tuber polysomal RNA was used as a control (T)
SUMMARY AND DISCUSSION

Three abundant proteins of approximate molecular weights of 22, 23, and 24 kilodalton (kD) 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-kD protein were prepared. Immunoblot analysis showed that the 22-, 23-, and 24-kD proteins were immunologically related and that these proteins were present in tubers and as higher molecular weight forms in leaves, but were not detectable in stems, roots, and stolons. The ratios of amino acid composition of the three purified proteins were equivalent, and all three proteins had identical amino-terminal amino acid sequences that match the deduced amino acid sequence of an abundant tuber protein cDNA, p34021. We found that the deduced amino acid sequence of p34021, which codes for the 22-kD protein, had high homology to the trypsin inhibitor of winged bean.

To determine whether the purified 22-kD protein has a proteinase inhibitor activity, we used the proteinase inhibitor gel determination assay. This gel determination assay revealed that the 22-kD potato tuber proteins were potent inhibitors of serine proteinases. Two of the three purified proteins from the 22-kD family of potato tuber protein were potent inhibitors of both trypsin and chymotrypsin. The third purified protein with molecular weight of approximately 24 kD inhibited only trypsin activity. Aside from an artifactual explanation, these three proteins could be the products of distinct but related genes from the same multi-gene family (Stiekema et al., 1988) or the three different forms may arise
from co-or post-translational modification of a common gene product (Sonnewald et al., 1989; Shumway et al., 1970). Comparison of the amino acid sequence of the putative reactive sites of several members of the proteinase inhibitors with the deduced sequence of the 22-kD protein revealed that the 22-kD protein contains sequences which could potentially possess "double-headed" sites of inhibition, one against trypsin and the other against chymotrypsin.

Like other potato proteinase inhibitors, the genes coding for the 22-kD proteins were developmentally regulated in tubers and environmentally regulated in leaves. Wound induction of the genes coding for the 22-kD potato tuber proteins also was detected at the RNA level. In leaves, transcripts of the 22-kD protein family were detected 6 h after wounding and were highest after 12 h in locally wounded leaves. The strongest induction occurred systemically in nonwounded leaves in response to mechanical wounding. Cross-hybridization of a cDNA, p34021, which codes for the 22-kD tuber protein, with proteinase inhibitor I, and II cDNAs and a second family of 20-kD potato tuber cDNAs revealed no cross-homology. Members of this second group of 20-kD potato tuber proteins also exhibited wound-induction in leaves at the RNA level.

In this report, I purified three potato tuber proteins ranging in molecular weight of 22 to 24 kD by using several protein-chemical methods, and characterized these proteins by immunoblotting and amino acid sequence analysis. I also demonstrated that the 22-kD proteins were potent inhibitors of trypsin and/or chymotrypsin. The genes coding for the 22-kD proteins were developmentally regulated in tubers and environmentally
expressed in leaves in response to mechanical wounding. On the basis of these results, this study identified an entirely new class of potato proteinase inhibitors whose regulated expression is similar to proteinase inhibitors I and II.
LITERATURE CITED


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