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Original article

Purification of an isoform of patatin with antimicrobial activity against *Phytophthora infestans*

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Abstract

Phytophthora infestans (Mont.) de Bary is infamous as the causal agent of the late blight epidemic contributing to the Irish potato famine of the mid 19th century and remains agriculture's most destructive disease as new mutations and migrations confound control measures. In efforts to develop resistant varieties, a somatic hybrid (the Wisconsin J series) between potato (*Solanum tuberosum*) and a wild relative (*Solanum bulbocastanum*) has been found to convey durable resistance against the pathogen. We screened the total protein (100 μg ml^{-1}) of somatic hybrid varieties J138, J138A12, J101K12, J103K12, and J101K9 for in vitro spore germination inhibition of *P. infestans*. Since J138 exhibited maximum inhibition at 150 μg ml^{-1} in comparison to other varieties, we purified a 40 kD protein from J138 tubers by assaying its ability to inhibit spore germination in *P. infestans* spores. The highly purified protein was able to inhibit *P. infestans* spore germination by 70% at the 2.5 μg ml^{-1} concentration. The N-terminal sequence of this protein was found to have exact amino acid homology to patatin, the major storage protein of potato tubers. The inhibitory protein has the same molecular weight as patatin and cross-reacts with patatin antibodies. The infection of J138 plants with spores of *P. infestans* under greenhouse conditions showed that patatin is expressed in stem tissue 72 h after the plant is inoculated with field isolates of *P. infestans* (US8). In this communication, we report the purification, characterization and antifungal activity against spores of *P. infestans* of patatin-J from potato tubers.

Keywords: Antifungal protein; Late blight; Patatin; *Phytophthora infestans*; Potato

1. Introduction

Phytophthora infestans (Mont.) de Bary, a water mould (Oomycete), has the ability to infect plants, is a causal agent of late blight in potatoes, and has historically had a significant impact on global potato production. As potato has become extensively adapted as a major world crop, late blight remains one of its worst diseases [20]. The worldwide migration of new biotypes of late blight into potato and tomato crops in recent decades has confounded control measures [38]. Prior to the 1980s, late blight in potato crops consisted of one clonal lineage of mating type A1 in which sexual reproduction was precluded by the absence of the required A2 mating type beyond the center of diversity of the pathogen, located in the Toluca Valley, Mexico [4,29–31,40]. Worldwide dissemination of A2 biotypes exhibiting increased virulence and insensitivity to systemic fungicides such as US8 have prompted a resurgence in potato breeding programs, utilizing wild *Solanum* species for inherent late blight resistance components [21].

Plants have evolved defense mechanisms to actively protect themselves from various pathogens [13]. The defense system in plants includes protective physical barriers and an array of antimicrobial compounds and enzymes. Potato resistance to late blight has been studied as race specific and quantitative, and as non-race specific and qualitative [17]. Biochemical studies of the defense responses of potato have revealed an array of constitutive and induced enzymes that

Abbreviations: Lypoxygena, (LOX); Phospholipase A, (PLA).

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contribute to potato defense. For instance, pathogenesis-related (PR) proteins such as chitinases and 1,3-β-glucanase, and osmotin-like proteins that accumulate in infected potato tissues upon pathogen attack are involved in the degradation of fungal and bacterial cell walls. Smaller, constitutively expressed peptides in potato, such as snakin-1 and pseudothionin-*St1*, have been found to possess antimicrobial activity [2,24,26].

Patatin is a nonspecific lipid acyl hydrolase that accounts for approximately 40% of the total soluble protein in mature potato tubers, and it has potent insecticidal activity against the corn rootworm. Also, patatin isoforms purified from *P. infestans*-infected tubers have been reported to have beta,1,3-glucanase activity. However, to date no correlation has been made between the *P. infestans* infection and the observed antimicrobial activities. All known patatin isoforms have nonspecific lipolytic activity, catalyzing the hydrolysis of phospholipids, glycolipids and mono and diacylglycerols [8,31].

The induction of plant defense responses relies on initial recognition of a pathogen at the molecular level and subsequent downstream signal transduction events [27]. Race specific pathogen recognition is hypothesized to result from the direct or indirect interaction of the product of a dominant or semidominant plant resistance gene (*R*) with a product derived from the corresponding dominant pathogen avirulence (*Avr*) gene [27]. Subsequent signal transduction events coordinate the activation of an array of defense responses such that resistance is dependent upon the plant–pathogen recognition process [23]. The highly conserved leucine rich repeat (LRR) domains found in plant *R* genes are associated with the molecular level recognition of pathogens [13]. A strong example of race specific resistance is found in the protein coat of Potato Virus X, which is recognized by the *Rx1* and *Rx2* genes from *Solanum andigena* and *S. acaule*, respectively [1]. While incorporation of multiple, specific *R* genes from species with resistance to late blight (*S. demissum, S. stoloniferum*, and *S. chacoense*) has been successful in conveying potato resistance, rapid asexual cycling allows for rapid mutation of the pathogen, bypassing the plant recognition receptors (*R* genes) and allowing potato infection [7,12,36]. Incorporation of more durable and general resistance from other relatives into agronomically desirable cultivars has often been precluded by the incompatibility of sexual crosses due to ploidy levels and the associated endosperm balance number (EBN) of potato [32].

Solanum bulbocastanum, a wild potato relative indigenous to Mexico, is one such species that has long been noted for its general resistance under the extreme disease pressure of late blight in the Toluca Valley, Mexico [22]. Incorporation of resistance alleles from this wild species into *S. tuberosum* by traditional breeding methods has been hindered by the incompatibility of the diploid wild species with the tetraploid *S. tuberosum* potato cultivar background [6]. Somatic hybridization of *S. bulbocastanum* + *S. tuberosum* (the Wisconsin J series) has resulted in progeny that exhibit strong and durable field resistance to the many biotypes of *P. infestans* present in the Toluca Valley [21]. The J series is currently being utilized as a donor of resistance in potato breeding programs in the United States, although the nature of the durable resistance to late blight conveyed by *S. bulbocastanum* is not understood. To provide a biochemical insight into the nature of the J series' resistance to late blight, we have investigated the inherent antifungal components present in the J series tubers through a series of chromatographic separations and antimicrobial assays against *Phytophthora infestans* US8. Using this combined biochemical and antimicrobial activity approach we have purified a protein of approximately 40 kDa with antimicrobial activity against *P. infestans*. Interestingly, the N-terminus sequence of this protein has exact homology to patatin, a major tuber storage protein of potato, and was found to be induced in the stem tissue of J138 following inoculation with *P. infestans* US8.

2. Results

2.1. Antifungal activity of total tuber protein

In searching for antimicrobial proteins against *Phytophthora infestans* from resistant potato sources, we found that the total protein extracted from tubers of the J series had strong inhibitory activity against *P. infestans* spore germination. Crude protein was prepared from the tubers of five different J series genotypes to identify the potential inhibitory effect of endogenous potato proteins against spore germination and growth of *P. infestans*. As shown in Fig. 1, inhibition of sporangial and zoospore germination and growth (40–80%) was observed among the different J series crude protein treatments. Based upon its high inhibitory activity (80%), genotype J138 was selected for further purification to isolate the protein(s) involved.

2.2. Chromatography separations

Crude proteins were initially separated by basic and acidic properties utilizing an economy column (Bio-Rad) filled

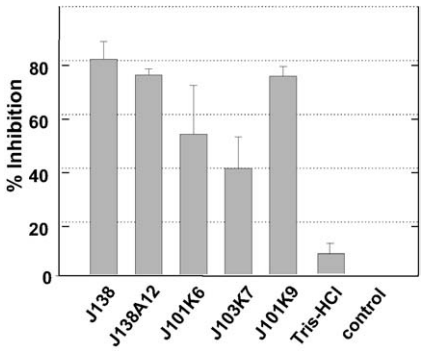


Fig. 1. Spore germination and growth inhibition of *P. infestans* US8. Sporangia were prepared as described in Section 4 and incubated with 150 μg ml^{-1} of crude protein extracted from tubers of the late-blight-resistant J series. Tris–HCl buffer (pH 7.0) and protein-untreated spores in growth media were used as controls.

Table 1
Purification of patatin J from tubers of potato J138. The protein was purified according the method described in Section 4. A spore germination inhibition assay was performed at each step of purification according to the method of Ali et al. [16] using 150 μg ml^{-1} of crude protein down to 2.5 μg ml^{-1} purified patatin. The sporangial suspension concentration was estimated using a cell counting chamber and adjusted to 1×10^5 sporangia ml^{-1}. For the germination assay, 100 μl of sporangiospore suspension and 50 μl of liquid Rye-A media was added to each well of a sterile 96-well titer plate. Tris–HCl (20 mM) without spores were taken as control

Steps of purification	Amount of protein purified (mg)	(%) Yield	*P. infestans* spore germination inhibition (%)
Ammonium sulphate precipitation (20–80%)	2386 (crude)	100	80% (150 μg ml^{-1})
Basic protein	547	22	No inhibition
Acidic protein	1695	71	60% (100 μg ml^{-1})
Anion-exchange chromatography (300–100 mM NaCl)	400	16.7	80–90% (10 μg ml^{-1})
Gel-filtration	20 (Patatin J)	0.8	70% (2.5 μg ml^{-1})

with diethylaminoethyl sephadex. The bound (acidic) and unbound (basic) fractions were collected and assayed for inhibitory activity (at 100 μg ml^{-1} of crude proteins. The results clearly indicated that the antifungal component(s) were present in the acidic fraction (Table 1) of the crude extract; these protein(s) were subjected to further anion exchange separation by fast protein liquid chromatography (FPLC).

To biochemically purify the antifungal protein from tubers of J138, crude proteins were subjected to anion-exchange and gel filtration chromatography separations, combined with testing the antifungal activity of collected fractions from each chromatographic step. As shown in Fig. 2, fractions 12 through 17 that eluted from an anion exchange column with a linear NaCl gradient exhibited maximum inhibition against spore germination (80–90%) at 10 μg ml^{-1}. The protein profile of these fractions, as visualized by SDS-PAGE and silver staining, revealed the presence of three proteins common to the fractions exhibiting higher levels of inhibition.

Fractions 12–17 were pooled together and then subjected to size exclusion chromatography utilizing a gel filtration column. Two peaks were collected as fractions 18 and 21 revealed a purified protein of ~40 kDa as detected by SDS-PAGE and coomassie staining (Fig. 3A,B). Inhibitory activity of the purified 40 kDa protein against *P. infestans* was assayed along with the total crude proteins from J138 for comparison (Fig. 3C). The results indicate that 2.5 μg ml^{-1} of the 40 kDa protein showed 70% spore germination inhibition in comparison to crude protein (150 μg ml^{-1}). The purified 40 kDa protein had an inhibitory effect 60 times stronger than the total J138 proteins (Table 1).

2.3. Biochemical analysis of purified antifungal protein

After chromatographic purification, the 40 kDa protein exhibiting inhibitory activity against the *P. infestans* spore

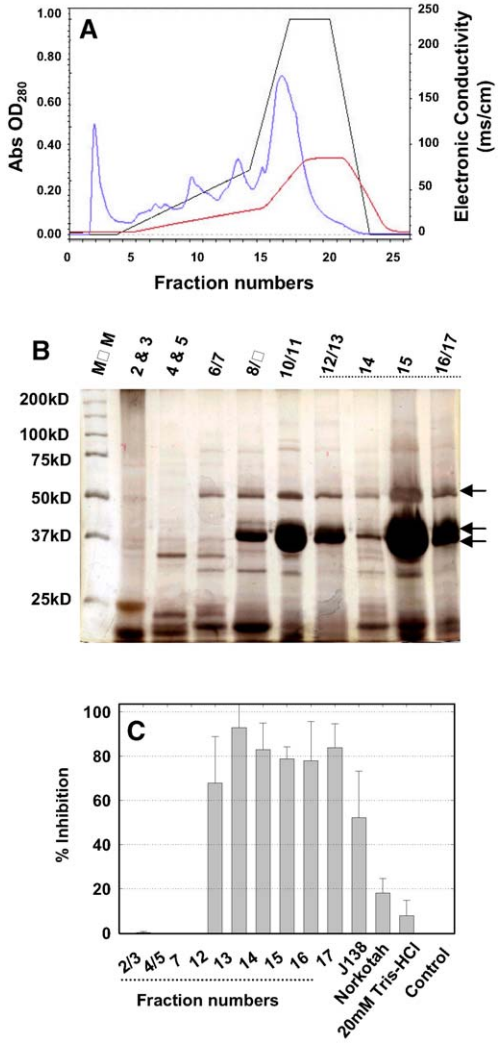


Fig. 2. Chromatographic separation and inhibitory activity assays of J138 tuber proteins. (**A**) Anion-exchange chromatogram. (**B**) SDS-PAGE of the collected fractions. Total tuber protein from J138 was subjected to an anion exchange column and eluted with a linear gradient of 300–1000 mM NaCl at 1 ml min^{-1}. Five microliters of each fraction were loaded onto the gel and visualized by silver staining. (**C**) Inhibitory activity of the collected fractions: 10 μg of each fraction was tested for inhibition of *P. infestans* spore germination as described in Section 4.

germination and growth was analyzed to determine the N-terminus amino acid sequence. The resulting 14 amino acid sequence, TLGEMVTVLSIDGG, was BLAST analyzed for sequence homology. This amino acid motif is fairly conserved in plant systems with patatin-like relatives [3] with upward of 75% homology with the patatin N-terminus motif reported in tobacco, Arabidopsis, sorghum, cowpea, cucumber, and rice (Fig. 4A). Our results showed that the N-terminus region of this purified protein had 100% homology with six reported N-terminus patatin isoforms from potato, *Solanum tuberosum*, as well as 100% homology with isoforms from *S. brevidens* (Fig. 4A) [3]. The N-terminus region of the purified protein represents the final gene product in which the signal peptide for vacuolar targeting of the storage protein has been cleaved post-translationally. Based on the homology of the N-terminal region of this antifungal

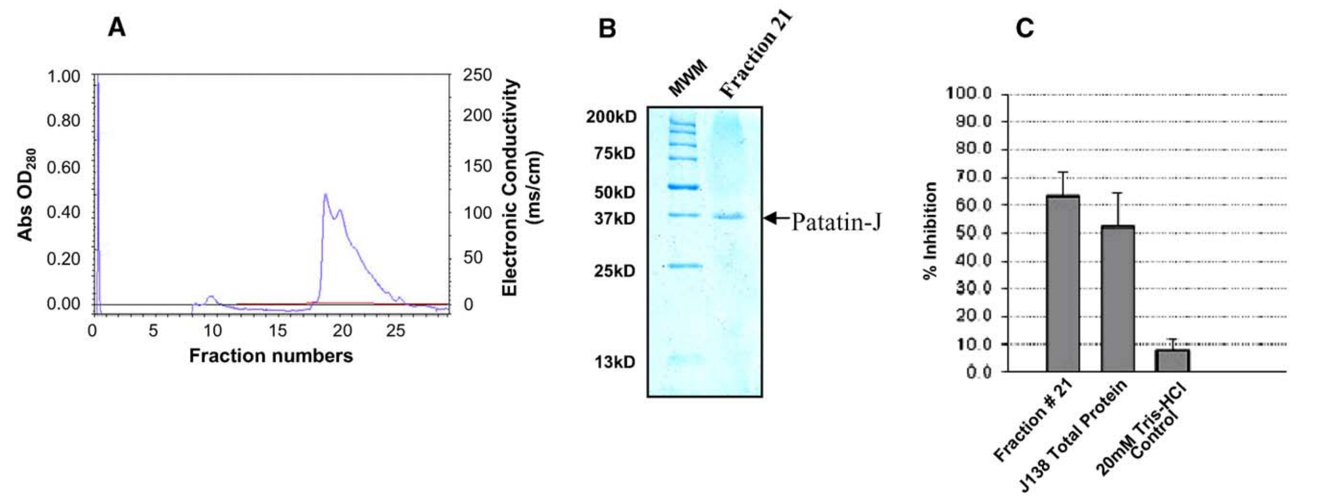
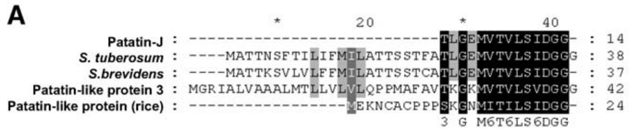


Fig. 3. Isolation of patatin-J and its inhibitory activity against *P. infestans*. (**A**) Size exclusion chromatographic separation of the Q protein fraction 21/22 as described in Section 4. The sample was injected into the column, eluted with a constant flow rate of 0.5 ml min^{-1} and 0.5 ml fractions collected. (**B**) SDS-PAGE of the purified patatin J in gel filtration fractions 21–23. Approximately 2 µg of protein was loaded onto the gel and stained with coomassie brilliant blue R-250. (**C**) Inhibitory activity of 5 µg J138 total protein and 2.5 µg ml^{-1} purified patatin J against *P. infestans*. Ten microliter 20 mM Tris–HCl buffer (pH 7.0) was used as a control.



B

Amino Acids	Patatin-J	Patatin Superior	Amino Acids	Patatin-J	Patatin Superior
Asx	9.22	7.93	□al	4.51	2.76
Thr	6.02	4.18	□et	1.96	0.88
Ser	5.20	4.45	□le	3.95	2.85
Glx	11.37	8.47	□eu	11.38	6.75
Pro	4.49	5.11	Thr	6.99	4.25
Gly	5.59	11.61	□is	6.77	5.68
Ala	5.49	3.71	□ys	1.80	1.57
Cys	0.00	16.37	Arg	4.85	3.56

Fig. 4. Biochemical characterization of patatin J. (**A**) Comparison of the N-terminal sequences of patatin J. Patatins isolated from *S. tuberosum* (Genbank accession no. CAA34169) and *S. brevidens* (no. AAA66198), a patatin-like protein 3 from tobacco (*Nicotiana tabaccum*, no. AAF98369), and a patatin-like protein from rice (*Oryza sativa*, no. BAC069505). (**B**) Comparison of amino acid profiles of patatin purified from J138 and cv Superior as percent composition of protein.

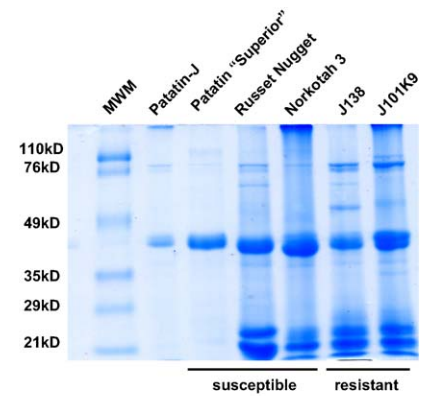


Fig. 5. Comparison of purified patatin isoforms and total tuber protein extracted from susceptible and resistant lines of potatoes. SDS-PAGE and coomassie staining of purified patatin from J138 (patatin J) and cv Superior compared to total tuber extracts.

protein purified from J138 tubers, we termed the protein "patatin J."

2.4. Comparison of patatin isoforms

All isoforms of patatin are approximately 40 kDa in size, immunologically indistinguishable, and possess extensive charge heterogeneity characterized by isoelectric focusing [16]. The molecular size of the purified patatin J was also compared with patatin purified from cv Superior and crude proteins of resistant J series tubers and tubers from susceptible cultivars using SDS-PAGE analysis (Fig. 5). Both purified isoforms were found to be approximately 40 kDa in size and similar bands were observed in all genotypes. To determine if the patatin isolated from the J series tubers possesses unique properties compared to patatin isoforms previously characterized, the percentage amino acid composition of patatin J was compared to patatin identified from *S. tuberosum* cv Superior. The amino acid results indicate approximately double levels of leucine, and 10 times the levels of lysine residues, in patatin J compared to patatin Superior (Fig. 4B). The variation of lysine, a polar amino acid, would explain the difference in the charge of patatin J compared to other isoforms. Patatin from cv Superior was found to have twice the amount of glycine residues. There was also a marked divergence in the percentage of cysteine residues between patatins analyzed; while this may be partially a result of inherent error in the analysis method, one may extrapolate a comparatively lower presence of cysteine residues in patatin J. These results strengthen our hypothesis that patatin J may be a new isoform with unique antimicrobial properties.

2.5. Expression of patatin in J138 plant tissue

The purified 40 kDa protein was further characterized by using total tuber protein of the resistant J series (J101K7, J138) and susceptible cultivars (Russet Norkota 3, Russet Nugget), and purified patatin from cv Superior and putative patatin J by doing western blots using patatin-superior polyclonal antibodies. The antibodies reacted with patatin J and showed the same molecular size cross reaction bands from all total-tuber extracts as well as from both purified forms of patatin, confirming patatin J as a patatin isoform (Fig. 6A).

Although tuber-isolated patatin J shows antifungal activity against *P. infestans*, it is difficult to correlate the field resistance of J138 to the occurrence of this protein in the roots, because secondary infection notoriously spreads through the canopy, infecting leaves and stems (although infected tubers may be a source of primary inoculum). Thus we examined the possible localization of patatin J in the leaves and stems of J series plants by western blot analysis. Total protein was extracted from leaves and stems of greenhouse-grown J101, J138, *S. bulbocastanum* (the donor parent conveying resistance to the J series), and susceptible cvs Russet Norkota- selection 3, Russet Burbank, and Cherry Red. No patatin antibody reaction was detected any of the protein extracted from stems (Fig. 6B) or leaves (Fig. 6C).

Since the highly purified patatin from J-series potato tubers (patatin-J) exhibited inhibition of spore germination

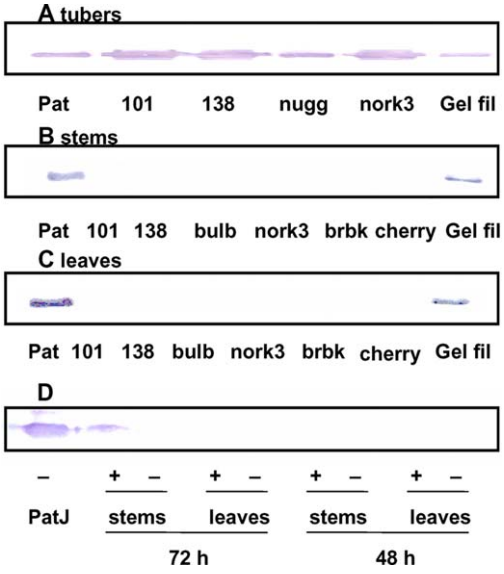


Fig. 6. Expression patterns of patatin in leaf, stem and tuber tissues from susceptible (nugg, Russet Nugget; nork3, Norkota 3; brbk, Russet Burbank; cherry, Cherry Red) and resistant (J138, J101K9, and bulb: *S. bulbocastanum*) lines. Approximately 5 μg of total proteins extracted from the tubers (**A**) the stems (**B**) and the leaves (**C**) of each potato line as described in Section 4 was run in 12.5% (w/v) SDS-PAGE, and western blot was performed using the polyclonal anti-patatin antibody as described in Section 4. (**D**) Localized induction of patatin J upon *P. infestans* infection. Stem and leaf tissues of J138 were collected 48 and 72 h after inoculation and subjected to western blot analysis. Plants inoculated (+) and not inoculated (–) with *P. infestans* were denoted, and purified patatin J from J138 tubers was used as a positive control.

(2.5 μg ml^{-1}/assay) of *P. infestans* spores under in vitro conditions, we wanted to know whether it works under in vivo conditions or not. For this, potato plants were inoculated with *P. infestans* spores under controlled greenhouse conditions. To our surprise, only the stems showed the induction of patatin after 72 h of inoculation, while we failed to get any induction in the leaf (Fig. 6D). These results indicate that patatin is expressed upon pathogen infection, but since all isoforms of patatin cross react with each other, it is impossible to tell if the induced protein is patatin-J or another patatin. In either case, the induction of patatin upon pathogen infection cannot be ruled out.

3. Discussion

Utilizing an in vitro bioassay to assess inhibition of spore germination of *Phytophthora infestans*, we have purified a 40 kD protein from potato tubers with strong antimicrobial activity against this pathogen. The purified protein showed amino acid homology to patatin and cross-reacted with anti-patatin antibodies. Patatin is one of the most abundant proteins in potato tubers, accounting for upwards of 40% of total soluble tuber protein, and has been found in every cultivated variety and wild species of *Solanum* examined [9,37]. All characterized isoforms of patatin are immunologically identical, yet differ in charge, purportedly attributed to post-translational glycosylation [37]. Major tuber storage proteins of other plant species such as sporamin of sweet potato (*Ipomoea batatas*), dioscorin of yam (*Dioscorea batatas*), and ocatin of the Andean tuber crop Oca (*Oxalis tuberosa*) have now been found to exhibit secondary biological functions such as insect feeding deterrent activity and/or antimicrobial activity [2]. These observations suggest that root/tuber storage proteins may have roles related to storage organ defense in addition to their primary nutritive function.

Additionally, all patatin isoforms examined have nonspecific lipolytic activity, catalyzing the hydrolysis of phospholipids, glycolipids, sulfolipids and mono- and diacylglycerols [8]. The role of this enzymatic activity has been explored in other organisms and extrapolated from similar patatin-like genes found in many other plant systems. In Arabidopsis, activation of patatin-like phospholipases by auxin or plant–pathogen interactions have been implicated in signal transduction and cross talk among signaling pathways, pointing to involvement in systemic acquired resistance [3]. Patatin-like phospholipases extracted from tobacco leaves infected with TMV are associated with fatty-acid-derived defense signaling during the hypersensitive response [3,33]. However, studies of the lipid hydrolase activity of patatin in potato defense mechanisms have been limited; it has been found to inhibit the growth of southern corn rootworm and western corn rootworm when fed to these insects in an artificial diet [19]. Recently, a patatin isoform purified from *P. infestans*-infected tubers has been reported to have β-1,3 glucanase activity [4]. Interestingly β-1,3 glucanase activity has been

implicated in the disruption of fungal cell walls and thus this enzyme is considered an antifungal protein. Although β-1,3 glucanase activity was identified in the reported patatin isoform, no correlation was made between this activity and potential antifungal roles. Our studies suggest that patatin J may potentially be involved in pathogenesis-related activity given the in vitro antifungal results.

Patatin is encoded by two classes of multigenic family [10]. Class I genes are expressed in relatively high amounts and are developmentally regulated and expressed exclusively upon differentiation of tubers from stem tissue. The class II genes, which differ from class I only by a 22 bp region in the untranslated 5′ region, are expressed predominantly in root tips [40]. In this study, western blot analysis of tubers, stems, and leaf tissue from resistant and susceptible lines showed that patatin is not detectable in leaves or stems of any examined genotypes, and, quite expectedly, did show patatin in all tubers examined. Considering the tissue specificity of patatin expression, biological issues regarding the epidemiology of resistance of J138 to late blight should be addressed. *P. infestans* spores characteristically infect and rapidly spread through the leaves of the potato canopy, subsequently infecting the entire plant, including tubers. Late blight-resistant J138 tubers were observed in the field to control the disease progression in the leaf stage [21]. However, we were unable to detect constitutive patatin J expression in the leaves and stems of J138 plants. This observation raises the question of whether patatin J could be contributing to resistance. Our studies found that patatin was detected by western blot analysis 72 h after inoculation of J138 plants with *P. infestans* in stems, and was not detectable in leaf and stem tissue of non-inoculated plants grown under the same greenhouse conditions.

Oxylipin profiling in pathogen-infected potato leaves has shown that plants respond to pathogen attack with a multicomponent defense response. Gobel et al. [5] have shown that synthesis of oxylipin via the lipoxygenase (LOX) pathway is one of the important mechanisms for the establishment of resistance in a number of pathosystems. Additionally, they have shown that, in potato cells, pathogen-derived elicitors preferentially stimulate the 9-LOX dependent metabolism of polyunsaturated fatty acids. However, this stimulation was caused only by the interaction of *Pseudomonas syringae* pv. *maculiosa* with potato plant, which does not lead to a disease, but not by *P. infestans*, the casual agent of late blight in potato. In the earlier studies, it was shown that in the interaction of potato plants with specific races of *P. infestans*, 9-LOX-derived divinyl ethers accumulated faster and to higher levels in the leaves of resistant plants than in those of susceptible ones, while 9-LOX gene expression was reported not to be significantly different [18]. Similarly all phospholipase A isolated and cloned from Arabidopsis belongs to the patatin group [3]. The involvement of phospholipase A in the plant signal transduction was reported by Holk et al. [3]. Plant PLA is a vacuolar enzyme and apart from its role in auxin signal transduction it has also been shown that it gets activated by pathogen and elicitors and it hydrolyzes phosphatidylcholine within 1–5 min to generate free fatty acids and lysophospholipids as potential second messengers [14,15,39]. All these reports suggest an unequivocal involvement of patatin and patatin-like proteins in the defense response of plants. Even though patatin-J is localized in the tuber, it clearly shows antifungal activity against the spores of *P. infestans*, and we cannot rule out the involvement of patatin-J in the more complicated process of triggering signal transduction upon pathogen attack and thus making the leaves also resistant to the late blight disease.

Studies of the induction of tuberization in potato have revealed that tubers can be induced in axial buds of stems following mechanical damage and disease infection in the field, from stem cuttings under short photoperiod conditions, and high sucrose levels in vitro [11]. Under these conditions, patatin expression has been observed in stems and petioles, but never in leaves. Based upon previous induction studies of patatin and studies of plant responses to pathogen infection, a possible mechanism of plant–pathogen interaction to produce patatin expression may be extrapolated. Soluble sugars have been found to activate PR proteins and defense related genes in plants [28]. Increased carbohydrate levels of leaves infected by pathogens have been observed, as well as increased accumulation of invertases and sucrose synthase following bacterial and fungal infection [18]. The rapid induction of sucrose-metabolizing enzymes is required to shunt carbohydrate resources into defense responses as well as other physiological responses to abiotic stress [34], and may turn on the expression of patatin in leaves. The results presented in this paper may provide useful information to control the causal agent of late blight of potatoes.

4. Methods

4.1. Biological materials

Greenhouse-grown potato tubers and in vitro plant materials of potato cultivars Russet Burbank, Russet Norkota selection 3, and the Wisconsin J series (J138, J138A12, J101K6, J101K9, and J103K7) were utilized for protein extractions [21]. In vitro plantlets were subcultured and acclimated to greenhouse conditions for protein extraction from leaf and stem tissues. *Phytophthora infestans* US8 cultures were provided by Dr. ASN Reddy (Colorado State University, Ft Collins, CO). Cultures were grown under dark conditions on Rye B media at 19 °C. Purified patatin from *S. tuberosum* cv Superior and polyclonal anti-patatin antibodies were graciously provided by Dr. David Hannapel (Iowa State University, Ames, IA).

4.2. Protein extraction from tubers

Potato tuber extracts were prepared by macerating 100 g of tuber tissue per 300 ml of protein extraction buffer

(250 mM NaCl, 10 mM EDTA, 10 mM Thiourea, 5 mM DTT, 1 mM PMSF, and 5% [w/v] polyvinylpolypyrrolidone) in 20 mM Tris–HCl (pH 7.0), in a blender (Waring, New Hartford, CT). The slurry was then centrifuged at 10,000 × *g* for 30 min., and the supernatant was brought to 20% ammonium sulfate followed by centrifugation at 11,000 × *g* for 30 min. The supernatant was collected and dialyzed against 20 mM Tris–HCl buffer, pH 7.0. The crude protein extract was concentrated to 5 mg ml^{-1} utilizing a YM 1 membrane in an Amicon stirred ultra filtration cell (Millipore, Bedford, MA). Protein concentration was estimated by the Bradford [25] method (protein estimation kit from BIO-RAD).

4.3. Protein extraction from leaf and stem tissues

Approximately, 1 g each of leaf tissue (with midvein removed) and stem tissue was immersed in liquid nitrogen, ground into a powder using a mortar and pestle, and suspended in protein extraction buffer as previously described. The solution was centrifuged at 10,000 × *g* for 35 min, and the supernatant was collected. Protein was concentrated by ammonium sulfate precipitation and estimated by the Bradford [25] (protein estimation kit from BIO-RAD) as previously described.

4.4. Sporangiospore germination bioassay

The sporangia germination bioassay was conducted according to the method of Ali and Reddy [16]. Sporangia were harvested from 3 to 4-week-old cultures of *P. infestans* by rinsing the plates with 5 ml sterile distilled water. The sporangial suspension concentration was estimated using a cell counting chamber and adjusted to 1×10^5 sporangia ml^{-1}. The spore suspension was then placed in a 4 °C cold treatment for 4 h to induce the release of zoospores. For the germination assay, 100 μl of sporangiospore suspension and 50 μl of liquid Rye-A media was added to each well of a sterile 96-well titer plate (Nalge Nunc International, Rochester, NY). Extracted potato tuber proteins (total and purified) and control treatments of 20 mM Tris–HCl buffer (pH 7), were applied in replicates of three wells per treatment (150 μg ml^{-1})]. Plates were covered with sterile lids and placed in a sealed polystyrene box with moistened filter paper to maintain humidity and incubated in the dark at room temperature. Optical Density (OD) readings were taken at 630 nm over a 72-h time course by a 96-well titer plate reader (Opsys MR, Dynex Technologies, UK). Net fungal growth was measured as the initial OD reading subtracted from the final OD reading. Inhibition of fungal growth was evaluated as the difference between net growth in the treated and untreated control, divided by the net growth in the untreated control × 100% to produce a percentage of fungal inhibition measurement. The mean inhibition of replicates within each treatment is reported with standard deviation.

4.5. Protein purification—chromatographic separations

The total protein was fractionated on an Uno-Q2 column (Bio-Rad, Hercules, CA) attached to a BioLogic Duo-Flow fast protein liquid chromatography system (Bio-Rad). Equilibration and loading of the protein were carried out in 20 mM Tris–HCl (pH 7.0), at a flow rate of 1 ml min^{-1}. The total protein was eluted using a linear gradient of 300–1000 mM NaCl at 1 ml min^{-1} for 27 min and 2 ml fractions were collected. Eluted fractions exhibiting strong antifungal activity were further purified by a longer gradient of 300–1000 mM NaCl over 46 min. Fractions exhibiting antifungal activity were then further purified by size exclusion using a Bio-Sil ® SEC 250 column (Bio-Rad) at a constant flow rate of 0.5 ml min^{-1} and 0.5 ml fractions were collected.

4.6. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed with 10% (w/v) acrylamide gels (9×) using an electrophoresis cell Mini-Protein II (Bio-Rad) according to the manufacturer's instructions [35]. High molecular weight protein markers (31–200 kD; Bio-Rad) were run simultaneously for each electrophoresis gel. The protein bands were visualized by coomassie blue or silver staining.

4.7. Electrophoresis and western-blot analysis

Proteins were electroblotted to nitrocellulose membranes using a Mini Trans-Blot® electrophoretic Transfer Cell (Bio-Rad). The blot was then probed with protein A-purified polyclonal rabbit anti-patatin antibodies and the membranes were developed using an Opti-4CN™ Detection Kit purchased from Bio-Rad, following the manufacturer's instructions. A patatin antiserum titer of 1:2000 was used for protein extracted from tuber, leaf, and stem tissues.

4.8. N-terminus sequencing and amino acid analysis

The purified protein was N-terminally sequenced on a Precise Protein Sequencer System (Applied Biosystems, Foster City, CA) at the Macromolecular Resources Facility (Colorado State University). Amino acid analysis and composition were determined by the Protein Structure Core Facility at the University of Nebraska using an Applied Biosystems 420H (Foster City, CA).

4.9. Greenhouse inoculation of J138 plants

Inoculation of J138 plants with *P. infestans* was carried out in collaboration with Dr. Jeff Miller (University of Idaho experiment station, Aberdeen, ID). Plants of J138 were grown from tubers in 15 cm pots under greenhouse conditions to the fully expanded eight- leaflet stage. Field isolates of *P. infestans* US8 were grown on Rye B media for ten days at 19 °C. Sporangia were then harvested by rinsing with distilled water. The sporangial suspension was chilled for 2 h at 4 °C in dark conditions. The abaxial sides of detached leaflets (cv Russet Norkota) were inoculated with 1.5 cm discs of sterile Whatman filter paper saturated with the spo-

rangial suspension. The leaflets were then placed on a mesh screen positioned above moistened paper towels in a sealed polystyrene box and incubated under a 16-h light treatment at 15 °C for 6 days. Infected leaflets were rinsed with distilled water and harvested sporangia were quantified using a hemacytometer (American Optical, Buffalo, NY). The sporangial suspension was adjusted to 1×10^4 sporangia ml^{-1} and incubated at 4 °C for 2 h to induce zoospore release. Whole plants sprayed with the spore suspension were placed in a plastic tent with humidifiers to maintain 100% relative humidity for 24 h. The plants were then removed from the tent and monitored in the greenhouse for disease development. Three replicates of stem and leaf tissue were collected from three separate inoculated and control (not inoculated) J138 plants at time intervals of 1, 12, 24, 48, 72 h, and 14 days. The samples were flash frozen in liquid nitrogen and stored at –80 °C for protein extraction and western blot analysis.

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