Purification and Characterization of $\beta_2$-Tomatinase, an Enzyme Involved in the Degradation of $\alpha$-Tomatine and Isolation of the Gene Encoding $\beta_2$-Tomatinase from Septoria lycopersici

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Lycopersicon species often contain the toxic glycoalkaloid $\alpha$-tomatine, which is proposed to protect these plants from general microbial infection. However, fungal pathogens of tomato often are tolerant to $\alpha$-tomatine and detoxification of $\alpha$-tomatine may be how these pathogens avoid this potential barrier. As an initial step to evaluate this possibility, we have purified to homogeneity a $\beta$-1,2-D glucosidase from the tomato pathogen Septoria lycopersici that hydrolyzes the $\beta$-1,2-D glucosyl bond on the tetracascaride moiety of $\alpha$-tomatine to produce $\beta_2$-tomatine. The enzyme is a 110-kDa protein with a $\alpha_4$ of 4.5 and a $K_m$ for $\alpha$-tomatine of 62 $\mu$M. Little or no activity was detected on a variety of other glycosides. The gene encoding this protein was isolated and contains an open reading frame of 803 amino acids that shares sequence homology with several other $\beta$-D-glucosidases. When S. lycopersici was incubated with $\alpha$-tomatine, $\beta_2$-tomatinase mRNA accumulated, suggesting that the enzyme is substrate inducible. Aspergillus nidulans expressed “$\beta_2$-tomatinase” activity when transformed with this gene but transformants were still sensitive to $\alpha$-tomatine.

Additional keywords: glucosidase, phytoanticipin, saponins.

$\alpha$-Tomatine (Fig. 1), a steroidal glycoalkaloid found in the tomato plant and other members of the family Solanaceae (Roddick 1974), belongs to a class of compounds commonly known as saponins. Saponins consist of an aglycone, either steroidal or triterpenoid in structure, linked to one or more sugars (Price and Fenwick 1987). Most saponins exhibit soaplike properties in water, possess hemolytic and cholesterol-binding properties, and exhibit significant antifungal activity (Price and Fenwick 1987). The presumed fungitoxic effects of $\alpha$-tomatine and other saponins are due to their interaction with $\beta$-3-hydroxy sterols, which results in increased membrane permeability and causes leakage of electrolytes (Keukens et al. 1992). Saponins such as $\alpha$-tomatine can accumulate to very high concentrations (up to 5% fresh wt.) in healthy plant tissue (Jadhav et al. 1981) and it has long been suggested that these compounds may be a general barrier to protect plants against microbial diseases and insect predation (Arneson and Durbin 1968a; Gallardo and Boehl R. 1990). Recently, the term phytoanticipin has been proposed to describe saponins and other low molecular weight antimicrobial compounds that are produced as part of normal plant development and that might function to protect plants from disease (VanEtten et al. 1994). This term was introduced in order to distinguish these passively produced antimicrobial compounds from phytoalexins, antimicrobial compounds that are synthesized de novo in response to microbial infection.

Although saponins are widely distributed throughout the plant kingdom (Price and Fenwick 1987), it is only recently that they have been shown to function as a deterrent to microbial infection (Bowyer et al. 1995; Osbourn et al. 1994). Avenacin, a triterpenoid saponin, was examined for its role in plant resistance against the fungal pathogen Gaumunnomyces graminis var. tritici. Avenacin is normally found in the outer layers of roots of oats (Avena sativa L.) but not in wheat (Triticum aestivum L.). Gaumunnomyces graminis var. tritici is a root rot pathogen of wheat but not of oats (Avena sativa L.) while G. graminis var. avenae is pathogenic on both. A correlation exists between the greater tolerance of G. graminis var. avenae to avenacin compared with that of G. graminis var. tritici, and its ability to detoxify avenacin by expression of a $\beta$-glucosidase that removes the $\beta$-1,2 and $\beta$-1,4-linked D-glucose molecules from the glycoside moiety of avenacin (Crombie et al. 1986; Turner 1961). The gene encoding this enzyme (“avenacinase”) was recently cloned and, using transformation-mediated gene disruption methods, mutants of G. graminis var. avenae that lack avenacinase activity were constructed (Bowyer et al. 1995). These mutants, while retaining their ability to cause disease on wheat, were no longer pathogenic on oats, establishing that detoxification of avenacin is important for the pathogenicity of this variant of G. graminis on oats. Furthermore, a variety of oats was identified that lacks avenacin in its roots and is susceptible to G. graminis var. tritici, further supporting a role for this phytoanticipin in disease resistance (Osbourn et al. 1994).

Historically, a similar scenario has been proposed for the involvement of $\alpha$-tomatine in disease resistance of tomato and for the importance of the detoxification of $\alpha$-tomatine in

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pathogenicity on plants that synthesize this phytoanticipin. Arneson and Durbin (1968b) demonstrated that fungi that are not normally pathogenic on plants producing α-tomatein are more sensitive to α-tomatein than are pathogens of these plants. They and others (Arneson and Durbin 1967; Ford et al. 1977; Pegg and Woodward 1986; Verhoef and Liem 1975; R. W. Sandrock and H. D. VanEtten, unpublished) demonstrated that most tomato pathogens produce enzymes that specifically degrade α-tomatein. Thus, it was suggested that α-tomatein may play a role in the plant's defense toward fungal saprophytes and nonpathogens of tomato and that detoxification of α-tomatein may be important for tomato pathogens. A different approach was followed by Defago and coworkers (Defago and Kern 1983; Defago et al. 1983) to evaluate the role of α-tomatein in disease resistance. They induced mutants of Nectria haematococca that were more tolerant to α-tomatein and demonstrated that these mutants were now pathogenic on green tomato fruits, which contain high levels of α-tomatein. The wild-type strains were pathogenic only on ripe fruit, which contain little or no α-tomatein.

As a means to further evaluate the role of α-tomatein in resistance and the role of its detoxification in pathogenicity, we have purified and characterized the “tomatinase” enzyme from the fungus Septoria lycopersici, a foliar pathogen of tomato. This enzyme was first described by Arneson and Durbin (1967), who demonstrated that degradation of α-tomatein by S. lycopersici occurs by the hydrolysis of the β-1,2-D glucosyl bond on the tetrasaccharide moiety resulting in the formation of the less toxic compound β2-tomatin (Fig. 1) (Arneson and Durbin 1968). β2-Tomatinase is not able to form a stable complex with membrane-bound sterols (Arneson and Durbin, 1968). Partial purification of the S. lycopersici β-1,2-D-glucosidase (EC 3.2.1.21) was achieved (Durbin and Uchytíl 1969) and we report here the purification to homogeneity of this enzyme which we call “β2-tomatinase.” The nomenclature given to this enzyme, β2-tomatinase, is designed to distinguish this enzyme from other fungal “tomatinases” that are known to hydrolyze the entire tetrasaccharide moiety or the other sugar molecules from α-tomatein (Schlösser 1975; Ford et al. 1977). During the purification and characterization of β2-tomatinase, we were informed that a partial cDNA encoding β2-tomatinase had been cloned from a different isolate of S. lycopersici by its homology to the avenacin gene from G. graminis var.avenae (Paul Bowyer and Anne Osbourn, personal communication). The sequence of an internal peptide from our purified enzyme matched identically to a segment of the deduced polypeptide sequence of this cDNA. This partial cDNA (and later the sequence of the full-length cDNA) was supplied to us by Anne Osbourn and Paul Bowyer and we used it to clone the genomic copy of β2-tomatinase from S. lycopersici, the properties of which are reported here.

RESULTS

Extraction and purification of β2-tomatinase.

Initial attempts to purify β2-tomatinase from culture filtrates were hampered by the secretion of melanin-like pigments when S. lycopersici was grown in liquid cultures. Cleavage of α-tomatein to β2-tomatin by cell-free extracts of such cultures was detected by thin layer chromatography (TLC), which verified that β2-tomatinase was present in these culture filtrates. However, when starting with culture filtrates as a source of the enzyme, the co-purification of these pigments through many of the steps hampered the purification of β2-tomatinase by interfering with protein quantitation and activity assays. Addition of tricyclazole, an inhibitor of melanin biosynthesis, to the cultures did not prevent pigment formation and the use of polyvinylpyrrolidone to remove the pigments from the culture filtrates was also unsuccessful. To overcome this problem, β2-tomatinase activity was readily detected, without accompanying pigments, in intracellular extracts of S. lycopersici mycelium and this source was used as the starting material for the purification of β2-tomatinase.

β2-Tomatinase was purified using a four-step process. Ammonium sulfate precipitation was used as an initial step in the enzyme purification from intracellular extracts. Protein precipitating in the 50 to 80% ammonium sulfate fraction contained the majority of the β2-tomatinase activity and, after dialysis in 25 mM Tris-HCl pH 7.5, 25 mM NaCl, was applied to a Mono Q anion exchange column. The β2-tomatinase activity eluted from the column between 100 mM and 125 mM NaCl (Fig. 2A). For the third step, the fractions containing activity were pooled and ammonium sulfate was added to 1.3 M. This was loaded onto a phenyl agarose column, which separates proteins based on their hydrophobic interaction with the column resin. β2-Tomatinase activity eluted from this column between 1.1 M and 1.0 M ammonium sulfate (Fig 2B). The final purification step involved gel filtration using a Superose 12 column. β2-Tomatinase activity eluted at approximately 140 kDa as determined by the elution times of proteins with known molecular weights (Fig. 2C).

Protein in the pooled fractions from each purification step were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie stain (Fig. 3). The sample from the Superose 12 column was found to consist of a single protein band with a molecular mass of 110 kDa. This purification scheme resulted in a 478-fold purification of β2-tomatinase (Table 1).

Characterization of β2-tomatinase activity.

β2-Tomatinase is a fairly stable enzyme and retained partial activity when incubated at temperatures up to 55°C for 15 min. The enzyme was active between a pH range of 4.5 to 8.5 but due to the low solubility of α-tomatein above a pH of 5.0, the optimum pH for activity could not be determined. Based on the elution profile from a Mono P column, a chromatofocusing column, the enzyme has a pl of 4.5.

Fig. 1. Structure of α-tomatein [(O-β-D-glucopyranosyl-(1,2 glu)-O-β-D-xylpyranosyl-(1,3 glu)-O-β-D-glucopyranosyl-(1,4 gal)-β-D-galactopyranosyl-tomatinide)]. Degradation of α-tomatein to β2-tomatin by the β2-tomatinase enzyme of Septoria lycopersici (β-1,2-D glucosidase, EC 3.2.1.21) results in hydrolysis of the β-1,2-glycosidic bond at (1).
The \( K_m \) of \( \beta_2 \)-tomatinase was determined using the enzyme fraction purified from the Superose 12 column. Because of the low substrate concentration required to obtain the \( K_m \), the more sensitive high-pressure liquid chromatography (HPLC) assay was used to measure the release of glucose from \( \alpha \)-tomatine in these experiments. The enzyme had normal saturation kinetics and a \( V_{\text{max}} \) at 900 \( \mu \)M \( \alpha \)-tomatine. The Hanse-Wolff method (Hanes 1932) was used to calculate \( K_m \) values; it was found that \( \beta_2 \)-tomatinase has a \( K_m \) value of 62 \( \mu \)M for \( \alpha \)-tomatine.

The substrate specificity of \( \beta_2 \)-tomatinase was evaluated by incubating the enzyme with other saponins that had \( \beta_1 \)-2 glycosidic linkages as well as with other simple \( \alpha_1 \) or \( \beta_1 \)-glycosides and comparing the enzyme’s glycosidic activity on these compounds with its activity on a similar molar concentration of \( \alpha \)-tomatine. For these assays, the HPLC assay for released sugars was again employed in order to detect low levels of activity. \( \beta_2 \)-Tomatinase shows a strong preference toward \( \alpha \)-tomatine, having a relative activity of 2% on F-

![Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the different preparations that had \( \beta_2 \)-tomatinase activity during the purification of \( \beta_2 \)-tomatinase. Lane 1, GIBCO-BRL high molecular mass markers, lane 2, 30 \( \mu \)g of starting mycelial protein extract; lane 3, 30 \( \mu \)g of protein from 50 to 80% ammonium sulfate precipitation; lane 4, 20 \( \mu \)g of protein with \( \beta_2 \)-tomatinase activity from Mono Q column; lane 5, 5 \( \mu \)g of protein with \( \beta_2 \)-tomatinase activity from phenyl agarose column; and lane 6, 5 \( \mu \)g of protein with \( \beta_2 \)-tomatinase activity from Superose 12 column. The gel was stained with Coomassie blue R-250.]

### Table 1. Purification of \( \beta_2 \)-tomatinase from *Septoria lycopersici*

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (umole glucose/min)</th>
<th>Specific activity (umole glucose/min/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2289</td>
<td>442</td>
<td>0.193</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>768</td>
<td>264</td>
<td>0.344</td>
<td>1.8</td>
<td>60</td>
</tr>
<tr>
<td>Mono Q</td>
<td>9.4</td>
<td>205</td>
<td>21.8</td>
<td>113</td>
<td>46</td>
</tr>
<tr>
<td>Phenyl agarose</td>
<td>5.0</td>
<td>201</td>
<td>40.6</td>
<td>210</td>
<td>45</td>
</tr>
<tr>
<td>Superose 12</td>
<td>1.3</td>
<td>120</td>
<td>92.3</td>
<td>478</td>
<td>27</td>
</tr>
</tbody>
</table>

* Samples from each chromatographic step were dialyzed in 25 mM Tris pH 7.5, 25 mM NaCl prior to activity assays.
ginitin and 0.4% on digitonin compared with its activity on α-tomatine. No activity was detected on the steroid-like or triterpenoid compounds ginitin, solasoline, and β-escin or on the glycosides stevioside, phlorizin, melezitose, celluliose, maltose, and sophorose.

**Protein sequence analysis.**

Internal peptides were generated for sequence analysis using Arg-C protease after several attempts to obtain N-terminal sequence data were unsuccessful. Two prominent peptides of 25 kDa and 43 kDa were detected in the digest after electrophoresis on a 12% SDS acrylamide gel and staining with Coomassie blue. These two peptides were electroeluted from the gel and sequenced. The N-terminal sequence of the 25-kDa peptide was ILGGFKMEKNQPDPQ while the sequence of the 43-kDa peptide was KRTESVGGVRQYLLS. Comparison of these sequences with the EMBL and GENBANK databases using the BLASTP search program of the University of Wisconsin GCG software (Devereux et al. 1984) revealed that the 25-kDa peptide was 87% similar and 73% identical to the N-terminal sequence of several serine proteases (elastase, trypsin, thrombin, and kallikrein) and is presumed to be the N-terminal sequence of the Arg-C protease. No significant homology was observed between the 43-kDa sequence and sequences within the two databases. However, 100% homology was found between the N-terminal sequence of the 43-kDa peptide and a portion of an open reading frame of a cDNA clone from *S. lycopersici* that was isolated by its hybridization to the avenacinase gene of *G. graminis* var. *avenae* (Paul Bowyer and Anne Osbourn, personal communication).

**Isolation of the β₂-tomatinase gene.**

The gene encoding β₂-tomatinase was cloned by screening a genomic cosmid library of *S. lycopersici* using a partial cDNA clone (a gift from Anne Osbourn and Paul Bowyer). Probing of the cosmid library with this cDNA resulted in the isolation of cosmid pKB2Y#6.1. A 4.1-kb *PstI/BglII* fragment from this cosmid was subcloned into pPK1, called β₂TOM, and was transformed into *Aspergillus nidulans*. This clone conferred both intracellular (Fig. 4) and extracellular β₂-tomatinase activity to *A. nidulans* verifying that the β₂-tomatinase gene was present on this fragment.

**Properties of the β₂-tomatinase gene.**

Sequence of the 4.1-kb *PstI/BglII* fragment resulted in the identification of a 2.580-bp open reading frame that is interrupted by three introns (Fig. 5). The sequence of the *S. lycopersici* cDNA, as determined by Osbourn et al. (1996), is identical to the sequence found in this open reading frame and confirms the location of the three introns, which contain sequences that match the consensus intron/exon border sequences for fungal genes (Bruchez et al. 1993). Sequence analysis of the 5' upstream noncoding region of the β₂-tomatinase gene revealed no conserved CAAT or TATA boxes, as is true with many fungal genes. The deduced polypeptide possesses a mass of 84.8 kDa and an isoelectric point of 4.9. This molecular mass differs from that of the purified β₂-tomatinase, which was estimated to be 110 kDa. This is presumably due to post-translational modification of the polypeptide. The 15 amino acid residue sequence obtained from the internal peptide of the β₂-tomatinase enzyme is present in the deduced open reading frame as residues 481 to 495. When the 4.1-kb *PstI/BglII* fragment was used as a probe on a Southern blot containing *S. lycopersici* genomic DNA digested with *PstI* a single hybridizing band of 7.0 kb was observed, which agrees with the restriction analysis of the cosmid clone pKB2Y#6.1 and indicates that the β₂-tomatinase gene exists as a single copy in the genome (data not shown).

To determine whether the β₂-tomatinase gene is inducible by α-tomatine, we isolated total RNA from *S. lycopersici* at various times during its incubation in 900 μM α-tomatine, a concentration similar to that found in tomato leaves. An increase in the level of β₂-tomatinase mRNA was observed after...
A search of GENBANK with the deduced amino acid sequence of the \( \beta \)-tomatinase gene revealed that a high degree of similarity exists between this gene and several other microbial \( \beta \)-glucosidases. The highest degree of homology was observed with the avenacin gene of *G. graminis* var. avenae (Anse Osborn, unpublished). The amino acid identity and similarity between the two enzymes are 53 and 68%, respectively. Homology was also observed with the cellobiose degrading enzymes from the filamentous fungus *Trichoderma reesei* (Barnett et al. 1991), the yeasts *Candida pelliculosa*, *Kluyveromyces fragilis*, *Saccharomyces fibuligera*, and *Pichia capsulata* (Kohchi and Toh-e 1985; Raynal et al. 1987; Machida et al. 1988; Janbon et al. GENBANK accession #U16259), and the prokaryotes *Clostridium thermocellum*, *Butyrivibrio fibrisolvens*, *Cellvibrio gilva*, and *Ruminococcus albus* (Grånertz et al. 1989; Lin et al. 1990; Kashiwagi et al. 1993; Takano et al. 1992). The degree of amino acid homology between \( \beta \)-tomatinase and the other fungal \( \beta \)-glucosidases was 40 to 45% identical and 59 to 62% similar.
For the prokaryotic β-glucosidases there was 18 to 34% identity and 42 to 56% similarity with the β2-tomatinase amino acid sequence. Although homology is observed throughout the peptide sequences, there are several small regions of high homology, most notably the highly conserved region around the catalytically active aspartic acid residue, as determined by studies involving a covalently-linked inhibitor to the active site of β-glucosidase A3 from Aspergillus wentii (Bause and Legler 1980) (Fig. 7). These β-glucosidases recently have been classified as belonging to the family 3 glycosyl hydrodases based on their strong amino acid sequence similarities (Henrissat and Bairoch 1993). Although homology exists between these β-glucosidases and β2-tomatinase it is unknown whether they possess activity toward α-tomatin.

**Sensitivity of *S. lycopersici* and *A. nidulans* transformants expressing the β2-tomatinase gene to α-tomatin and β2-tomatin.**

*Septoria lycopersici*, *A. nidulans*, and four transformants of *A. nidulans* that express the β2-tomatinase gene were tested for their sensitivity toward α-tomatin and β2-tomatin by radial growth assays on medium supplemented with these compounds. At the highest concentration tested (1 mM) there was a 10% inhibition in the growth of *S. lycopersici* by α-tomatin and no reduction of growth on medium containing this concentration of β2-tomatin (data not shown). The ED₅₀ (effective dose whereby growth rate is reduced to 50% of that seen on control plates lacking the compound) of α-tomatin was 40 μM for all transformants of *A. nidulans* and their growth response to all concentrations was identical to that observed with untransformed *A. nidulans* (Fig. 8A). When plated on medium containing different concentrations of β2-tomatin, the ED₅₀ was 220 μM for untransformed *A. nidulans* and 120 μM for the transformants. Surprisingly, while both untransformed and transformed *A. nidulans* showed similar growth patterns on medium containing the lowest and two highest concentrations of β2-tomatin, the transformants were statistically more sensitive (*P = 0.05*) than untransformed *A. nidulans* at 100 μM and 150 μM β2-tomatin (Fig. 8B).

**DISCUSSION**

As a first step to determine the biological role of α-tomatin degradation by the tomato pathogen *S. lycopersici*, we have purified to homogeneity the "tomatinase" enzyme that was partially purified and characterized by Durbin and Uchytíl (1969) and have isolated the gene encoding that activity. The isolate used in this study and the one used by Arneson and Durbin both produce an enzyme, β2-tomatinase, that converts α-tomatin to β2-tomatin and the Km value we found for this enzyme on α-tomatin (62 μM) was similar to what they reported (60 μM). Further characterization of β2-tomatinase using glycodies with glycosidic linkages and/or steroid or triterpenoid saponins similar to α-tomatin demonstrated that this enzyme is highly specific toward α-tomatin. Such a strong preference toward α-tomatin and the low Km of this enzyme is consistent with the hypothesis that this tomato pathogen may have evolved a specific enzyme to detoxify this phytoalexin.

The genomic copy of the gene encoding β2-tomatinase was isolated from *S. lycopersici* (T512) using a partial cDNA from a different isolate of *S. lycopersici* identified by cross-hybridization to the avenacinase gene from *Gaetumannomyces graminis* var. *avenae* (Bowyer et al. 1995). The avenacinase protein has an enzymatic function similar to that of β2-tomatinase and is responsible for the detoxification of the oat phytoalexin, avenacin. When transformed into *A. nidulans* the genomic copy of the β2-tomatinase gene was able to confer both intracellular and extracellular β2-tomatinase activity.

Considerable sequence homology was observed between β2-tomatinase and other β-glucosidases belonging to the family 3 of the glycospyl hydrodases proposed by Henrissat and Bairoch (1993). It is unknown whether the enzymes belonging to this family, except avenacinase, possess saponin-degrading activity. However, many of the organisms found within this group, such as the ruminant bacteria or the soil-inhabiting saprophytes, may come in contact with saponins in nature and may possess these enzymes for detoxification, degradation of saponins as a food source, or for a commensal relationship between two organisms. The homology observed between β2-tomatinase and avenacinase implies that "tomatinases" from other tomato pathogens, or "saponinases" from pathogens found to parasitize plants containing saponins, may be isolated using probes or degenerate oligonucleotides of the conserved regions among these two enzymes. β2-Tomatinase activity is found in the tomato pathogens *Stemphylium solani* (R. W. Sandrock and H. D. VanEten, unpublished), *Verticillium albo-atrum* (Pegg and Woodward 1986), and *Alternaria solani* (Schlösser 1975; R. W. Sandrock and H. D. VanEten, 1986).

![Fig. 6](image.png)

Fig. 6. Northern analysis of the induction of the *Septoria lycopersici* β2-tomatinase gene. Total RNA was extracted from *S. lycopersici* incubated in the presence or absence of α-tomatin at various time points and 5 μg RNA from each time point was subjected to Northern analysis using the 4.1-kb *PstI*/*BglII* fragment containing the genomic copy of the β2-tomatinase gene as a probe. Lane 1, 0 h; lane 2, 2 h, buffer control; lane 3, 2 h, in the presence of α-tomatin; lane 4, 4 h, buffer control; lane 5, 4 h, in the presence of α-tomatin; lane 6, 8 h, buffer control; lane 7, 8 h, in the presence of α-tomatin; lane 8, 24 h, buffer control; lane 9, 24 h, in the presence of α-tomatin.
unpublished). Other fungal pathogens of tomato, such as Fusarium oxysporum f. sp. lycopersici (Ford et al. 1977) and Botrytis cinerea (Verhoef and Liem 1975), are known to produce an enzyme that cleaves the β-1,3 galactosidic linkage of α-tomatine releasing the tetrascarhide moiety of α-tomatine (refer to Figure 1). It is possible that this enzyme shares similar structural features and conserved amino acid sequences with β2-tomatinatease in regard to the portion of the enzyme that is specific toward the recognition of α-tomatine.

Previous research has led to the belief that removal of the glucose side chain to produce β2-tomatine or the release of the entire tetrascarhide to produce tomatidine from α-tomatine were detoxification reactions because the products formed were thought to be nontoxic (Arenson and Durbin 1968b). Our results with transformants of A. nidulans expressing β2-tomatinatease demonstrate that they are as sensitive to α-tomatine as was the fungus prior to transformation (Fig. 8A). However, this may be due to poor expression of the β2-
tomatinatease gene in the transformants. An additional enigma is why the transformants of A. nidulans expressing β2-tomatinatease are more sensitive to the intermediate levels of β2-tomatine than is the untransformed fungus (Fig. 8B). Although β2-tomatine cannot bind to β3-OH sterols, which are thought to be requisite for the toxic effects of α-tomatine (Arenson and Durbin 1968b), A. nidulans is still more sensitive to β2-
tomatine than S. lycopersici and many other fungal pathogens of tomato are (R. W. Sandrock and H. D. VanEtten, unpublished data). It is possible that β2-tomatine and α-tomatine share unknown properties that render them toxic to A. nidulans and some other fungi or that β2-tomatine has a unique mode of toxicity to these fungi.

Clearly, S. lycopersici is highly tolerant to both α-tomatine and β2-tomatine, even though it lacks the ability to degrade β2-tomatine. It may be that this fungus possesses additional tolerance mechanisms toward plant phytotoxins and polypeptide antibiotics that interact with sterols or membrane lipids are known to exist in several fungi (Denny and VanEtten 1983;

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**Fig. 7.** Highly conserved region among the family 3 β-glucosidases. Homology was maximized by introducing gaps within the sequences and is denoted by dots. Numbers to the right of the end amino acid denote the amino acid residue number from the N-terminus of the organisms' respective β-glucosidases. No residue number exists for the Aspergillus wentii sequence as it was isolated as a peptic fragment. Sly = Septoria lycopersici; Gga = Galleria mellonella; Tre = Trichoderma reesi; Sfi = Sclerotinia sclerotiorum; Cpe = Candida pelliculosa; Pca = Plectra capsulata; Kfr = Kluyveromyces fragilis; Cah = Clostridium thermocellum; Bfi = Butyrivibrio fibrisolvens; Cgi = Cellvibrio gilvus; RaI = Ruminococcus albus; Awe = Aspergillus wentii. The asterisk indicates the aspartic acid residue of A. wentii β-glucosidase A3 that binds the inhibitor conductol B epoxide.

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**Fig. 8.** Sensitivity of untransformed and transformed Aspergillus nidulans toward α-tomatine and β2-tomatine. Squares represent the growth curve of untransformed A. nidulans while circles represent the growth curve of transformant T4 and are indicative of the growth pattern of all the A. nidulans transformants. A, Growth patterns of untransformed and transformed A. nidulans on medium containing α-tomatine. B, Growth patterns of untransformed and transformed A. nidulans on medium containing β2-tomatine. Growth patterns were found to differ significantly between untransformed and transformed A. nidulans at 100 μM and 150 μM using a Student's t test (t0.05).
Defago and Kern 1983; Kasbekar and Papavinasasundaram 1992). It may be that A. nidulans lacks these nondegradative tolerance mechanisms.

Cloning of the gene encoding β-tomatinate now makes it possible to resolve some of these unknowns as it should be feasible to make β-tomatinate minus mutants in S. lycopersici through transformation-mediated gene disruption procedures. The properties of these mutants should clarify whether degradation of α-tomatine to β-tomatine by the enzyme is necessary and sufficient for S. lycopersici to parasitize tomato plants. We are currently developing a transformation system for S. lycopersici in order to carry out these experiments.

MATERIALS AND METHODS

Strains, plasmids and media.

Septoria lycopersici (isolate #T512) was supplied by Verna Higgins (University of Toronto, Department of Plant Pathology, Montreal, Canada). For long-term storage, spores were suspended in 25% glycerol and kept at −80°C. Pycnidiospores were used as inoculum for liquid medium. These were produced by growth at 24°C on 150 × 15 mm petri plates containing V8 agar medium (200 ml of V8 juice, 2 g of CaCO₃, 20 g of agar per liter) for 7 days. Pycnidiospores are only produced when S. lycopersici is grown on solid medium, but colonies of this fungus will not grow beyond approximately 5 mm in diameter. Thus, to insure the formation of numerous colonies for pycnidiospore production, 7 × 10⁴ spores were spread onto the 150-mm V8 agar plates. Optimal production of pycnidiospores occurred if the V8 medium was allowed to dry for 1 to 2 days after pouring the molten agar medium and if the plates were not wrapped with Parafilm after inoculation. After 7 days, spores were collected from plates and 1 × 10⁴ spores were used to inoculate 1 liter of yeast-sucrose broth (per liter: 17 g of sucrose, 3 g of yeast extract, 2 g of KH₂PO₄, 0.2 g of MgSO₄ pH 5.8) contained in a 2-liter size flask. Cultures were shaken at 200 rpm for 6 days at 24°C. Mycelium (approximately 50 g/liter) was harvested onto Miracloth (Calbiochem, La Jolla, CA) by vacuum and either used immediately for enzyme purification or stored at −20°C.

Aspergillus nidulans strain UCD1 was grown on Aspergillus medium supplemented with tryptophan, lysine, methionine, arginine, PABA (para-amino-benzoic acid), and biotin (Yelton et al. 1984). Either tryptophan or arginine was omitted from the transformation selection medium depending on which gene was used as the selectable marker during transformation.

Escherichia coli strain DH5α or DH5αMCR2 was used throughout the study and grown in Luria broth (LB) medium or on LB agar. Medium was supplemented when necessary with 100 μg ampicillin per ml.

Plasmids used in this study were pTOM1 containing the β-tomatinate cDNA (Anne Osbourn and Paul Bowyer), pBlueScriptSK− (Stratagene, La Jolla, CA), A. nidulans cosmid pKBY2 (Yelton, et al. 1985), and plasmid pPK1 carrying the A. nidulans ArgB gene (Tom Adams, Department of Biology, Texas A&M University).

Enzyme purification.

This protocol represents an optimized purification scheme based on the preliminary protein characterization described by Durbin and Uchytíl (1969) and our preliminary trials. All steps were carried out at 4°C unless otherwise stated. Routinely, 50 g of mycelium was homogenized for 1 min in 50 ml of 50 mM Tris-HCl pH 7.5, 50 mM NaCl, and 1 mM DTT and 50 g of glass beads (Sigma Chemical Co., St. Louis, MO) using a Bead Beater (Biospec Products, Bartlesville, OK). The homogenate was centrifuged for 30 min at 15,000 × g. Mycelial debris was discarded and (NH₄)₂SO₄ was added to 50% saturation. The supernatant was stirred for 1 h and then centrifuged for 45 min at 20,000 × g. The pellet was discarded and (NH₄)₂SO₄ was added to 80% saturation, stirred, and centrifuged as above. The pellet was resuspended in 100 ml of 25 mM Tris-HCl pH 7.5, 25 mM NaCl and dialyzed against 4 liters of the same buffer for 24 h with a buffer change after 10 h. Fifty milliliters of the dialyzed solution (200 ml total prior to dialyzation) was loaded onto a Mono Q 10/10 column (Pharmacia, Piscataway, NJ) at 4 ml/min using a Waters HPLC system (Millipore, Bedford, MA) and washed with the same buffer until the absorbance at 280 nm reached baseline. Proteins were then eluted with a 25 mM to 400 mM NaCl linear gradient (180 ml total volume) at 24°C and 3-ml fractions were collected. Fractions containing β-tomatinate activity were pooled and dialyzed overnight against 1 liter of 25 mM Tris-HCl pH 7.5, 25 mM NaCl. The pooled fractions were concentrated to 2 ml using an Amicon ultrafiltration cell and a YM-30 filter membrane (Amicon Inc., Beverly, MA). (NH₄)₂SO₄ was added to a final concentration of 1.3 M and the sample was applied to a phenyl agarose 2 × 5 cm column (Sigma) and eluted using a 1.3 M to 0.6 M (NH₄)₂SO₄ linear gradient at 0.4 ml/min (24 ml total volume). The active fractions were pooled and concentrated to 200 μl using an Amicon Centricon-30 microconcentrator, applied to a Superose 12 column (Pharmacia) and eluted at 0.4 ml/min with 25 mM Tris-HCl pH 7.5, 25 mM NaCl. Fractions of 50 μl were collected and active fractions were pooled and stored in 20% glycerol at −80°C.

Protein concentration was determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. Proteins were analyzed on 8% polyacrylamide SDS gels using a Hoeffer Mighty Small II (Hoeffer Scientific Instruments, San Francisco, CA) gel apparatus. GIBCO-BRL high molecular weight standards (Life Technologies, Inc., Gaithersburg, MD) were used to estimate protein size. Proteins were visualized by Coomassie staining (Merril 1990).

Enzyme assays.

Qualitative analysis of enzyme activity was determined by TLC. The enzyme was incubated in 500 μl of 250 μM α-tomatine (Sigma), 50 mM sodium acetate pH 5.0 at 37°C. The reaction was stopped by the addition of 25 μl of 30% ammonium hydroxide, which also served to precipitate the glycoalkaloids. After incubating the reaction on ice for 15 min the mixture was centrifuged at 3,000 × g for 15 min at 24°C. The supernatant was removed and the precipitated glycoalkaloids were dissolved in 50 μl of ethanol. Approximately 10 μg of the glycoalkaloids was spotted onto an aluminum-backed silica gel TLC plate (0.2 mm thick, Kieselgel 60 F₂₅₄, EM Separations, Gibbstown, NJ) and developed in ethyl acetate, methanol, acetic acid, and water at a ratio of 30:20:10:1. The TLC plate was allowed to dry and then was dipped in 30% sulfuric acid and subsequently placed at
110°C for 10 min. α-Tomatine was observed to have an $R_f$ value of 0.23 while that of β2-tomatine was 0.4. Quantitative analysis of enzyme activity was determined using a modified version of the alkaline ferricyanide reducing sugar assay (Park and Johnson 1949). Reaction mixtures contained 3 ml of 2.5 mM α-tomatine, 50 mM sodium acetate pH 5.0 and were incubated with enzyme at 37°C. Aliquots of 500 µl were taken at 0, 5, 10, 15, and 20 min and added to 750 µl of a solution consisting of 3.4 mM K3Fe(CN)6, 3H2O, 103 mM KCN, and 252 mM Na2CO3. Tubes were immediately placed in a boiling water bath for 10 min. The mixture was then placed on ice for 1 min and an absorbance reading was taken on a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 420 nm. Assays were done three times. One unit of β2-tomatinate is considered to be the amount of enzyme that catalyzes the formation of 1 µmole glucose per min at 37°C.

Characterization of β2-tomatinate activity.

Effect of temperature on enzyme activity was evidenced by incubating the enzyme at 24, 37, 45, 55, 60, and 65°C for 15 min. The enzyme was then tested for activity by placing it in 500 µl of 2.5 mM α-tomatine, 50 mM sodium acetate pH 5.0 at 37°C for 15 min. Activity was measured using the alkaline ferricyanide assay.

The pI of β2-tomatinate was determined using the chromatographic column Mono P (Pharmacia). Purified enzyme (100 µg) from the Superose 12 column was loaded onto the Mono P column in 25 mM bis-Tris-HCl pH 7.1, 25 mM NaCl and eluted with a 1:10 dilution of Polybuffer 74 (Pharmacia) in distilled water (pH adjusted to 4.0 using 1 M HCl). Enzyme activity was determined using the alkaline ferricyanide assay.

Enzyme kinetics.

Enzyme was added to 500 µl of 0.05, 0.1, 0.2, 0.25, 0.33, 0.6, 0.8, 1.0, 1.25, 1.67, 2.0, and 2.5 mM α-tomatine in 50 mM sodium acetate pH 5.0 and incubated for 10 min at 37°C. Reactions were stopped with NaOH (150 mM final concentration) and proteins were removed by partitioning the extract with chloroform at a ratio of 1:1 chloroform to reaction volume. The amount of glucose produced in the reaction mixture was determined using a Dionex (Sunnyvale, CA) HPLC system in conjunction with a Dionex CarboPac PA1 column. Reaction mixtures (50 µl) were injected onto the CarboPac column and eluted with 150 mM NaOH. Carbohydrate detection was by pulsed amperometric detection (Dionex Advanced PAD) at 35°C. For calibration standards and elution times, various concentrations of mono- and disaccharides were used. Substrate divided by velocity was plotted versus substrate concentrations and the experimental data were fitted to obtain the $K_m$ using Cricket Graph (Computer Associates, San Diego, CA). Reactions were done three times.

Substrate specificity.

Melezitose, β-escin, phloridzin, and stevioside were purchased from Sigma (St. Louis, MO). Solasonone and digitonin were purchased from Research Plus, Inc. (Bayonne, NJ). Sophorose was purchased from Roth (Atomergic Chemetals Corp., Plainview, NY). Gitonin and F-gitonin were gifts of Yutaka Sashida (Tokyo College of Pharmacy, Japan). Approximately 10 ng of purified was added to 500 µl of 2.5 mM α-tomatine, digitonin, solasonone, melezitose, β-escin, phloridzin, stevioside, sophorose, cellobiose, or maltose in 50 mM sodium acetate pH 5.0. Due to the low level of solubility of digitonin and solasonone 1% Tween 80 was added to reaction mixtures containing these compounds and also to α-tomatine as a control. Reactions in gitonin and F-gitonin were carried out at 100 µM concentrations and in 2% Tween 80 for the same reason as above. The amount of released sugar in all reaction mixtures was measured as done to determine the $K_m$ value.

Enzyme sequence.

Enzymatic digestion of β2-tomatinate was carried out as described by Stone and Williams (1993). Briefly, 100 µg of lyophilized tomatinate was dissolved in 50 µl 8 M urea, 0.4 M NH4HCO3, 4 mM DTT, and incubated at 50°C for 15 min. After cooling to room temperature, 5 µl of 100 mM iodoacetamide was added and the reaction incubated at room temperature for 15 min. The volume was increased to 200 µl and 5 µl of 1 µg of Arg-C (Sigma) per µl was added and incubated overnight at 37°C. Peptide fragments were separated by PAGE on a 12% SDS acrylamide gel. Peptides were visualized by Coomassie staining (0.1% Coomassie blue R-250 in 10% methanol, 0.5% acetic acid) and de-stained in 10% methanol. The acrylamide gel containing the individual peptides was cut from the gel and the peptides were electroeluted using a Hoeffer GE 200 Gel Eluter. The peptides were precipitated by incubation with 10% cold trichloroacetic acid (Sigma) for 30 min at 4°C, centrifuged for 15 min and dried at room temperature. Peptides were sequenced via Edman degradation by the Protein Sequencing Facility (University of Arizona, Tucson).

Recombinant DNA techniques and nucleic acids analysis.

Restriction enzyme digestions, subcloning procedures, Southern blot analysis, Northern blot analysis, and colony hybridization were carried out as described by Sambrook et al. (1989). A Qiagen kit (Qiagen Inc., Dusseldorf, Germany) was used for DNA isolation from agarose gels. Septoria lycopersici DNA was isolated from fungal mycelium according to the method described by Garber and Yoder (1983). Library construction was carried out according to Sambrook et al. (1989) and Yelton et al. (1985) using the cosmid vector pKBY2. Eleven cosmids clones were isolated from the library after hybridization with the S. lycopersici β2-tomatinate cDNA contained in pTOM1. A 4.1-kb Pestl/BglII fragment on which the β2-tomatinate gene resides was initially cloned from cosmids #6.1 as a 5.2-kb Pestl/XbaI fragment into pBluescriptSK+. This clone was then digested with Pestl and partially digested with BglII to obtain the 4.1-kb Pestl/BglII fragment that contains an internal BglII site. This fragment was then subcloned into the Pestl and BamHI sites of pBluescriptSK+ called pβ2Tom. In order to test expression of the β2-tomatinate gene in A. nidulans, the insert in pβ2Tom was excised by digestion with KpnI and XbaI and subcloned into the KpnI and XbaI sites of pPK1.

To analyze the transcriptional induction of β2-tomatinate mRNA, S. lycopersici was grown for 2 days. The mycelium was collected by vacuum filtration and washed in 25 mM sodium acetate pH 5.0. Approximately 5 g of mycelium was added to 50 ml of 25 mM sodium acetate pH 5.0 with or without 900 µM α-tomatine. Fractions (10 ml) were removed.
and total RNA was extracted by the method of Reinert et al. (1981). Degradation of α-tomatine in the culture filtrate was determined by TLC.

Screening of S. lycopersici DNA for β2-tomatine expression in A. nidulans.

Cosmid clones and subclones of cosmids that hybridized to the cDNA contained in pTOM1 were screened for expression of β2-tomatine activity in A. nidulans. Cosmids and plasmids were transformed into A. nidulans based on the protocol developed by Yelt et al. (1983). 2 x 10⁸ spores of A. nidulans transformants were inoculated in 50 ml of Aspergillus medium and grown for 2 days at 30°C at 250 rpm. Mycelium was collected by vacuum filtration and washed once with 50 mM sodium acetate pH 5.0, 50 mM NaCl and resuspended in 25 ml of the same buffer. Mycelium was ground using the Bead Beater and mycelial debris was centrifuged for 10 min at 15,000 x g at 4°C. One milligram of crude protein extract in 250 μl of 50 mM sodium acetate pH 5.0, 50 mM NaCl was added to 250 μl of 250 μM α-tomatine, 50 mM sodium acetate pH 5.0 and incubated at 37°C for 30 min, 1 h, and 3 h. The reaction was stopped and the products produced during the reaction were determined by TLC using the same methods described in the enzyme assays section.

Bioassay for sensitivity of fungi to α-tomatine and β2-tomatine.

Septoria lycopersici and A. nidulans were tested for their sensitivity to α-tomatine or β2-tomatine by aerial growth assays on medium supplemented with these compounds. An agarose plug (6 mm diameter) of actively growing mycelium of A. nidulans was placed onto medium containing 0, 10, 50, 100, 150, 300, or 500 μM α-tomatine or 0, 50, 100, 150, 200, 300, and 500 μM β2-tomatine and placed at 37°C. Radial growth was measured every 24 h for 3 days. Percent inhibition was determined by dividing the radial mycelial growth after 3 days on plates supplemented with α-tomatine or β2-tomatine by the radial mycelial growth on plates lacking these compounds. The concentration of compound that gave 50% inhibition of radial growth was extrapolated from these values. For S. lycopersici, an agarose plug was placed on medium containing 0, 0.08, 0.2, 0.4, 0.8, or 1.5 mM α-tomatine or 0, 1.0, and 1.5 mM β2-tomatine. Experiments were done three times.

DNA sequencing.

DNA sequencing was performed by generating a series of overlapping deletions using the Erase-a-Base System (Promega Corp., Madison, WI) in pBluescruptSK+ and sequencing by the deoxy chain-termination method using the M13 universal (~20) and M13 reverse sequencing primers, Sequenase (United States Biochemicals, Cleveland, OH) and deoxyadenosine 5’-of[33P] thiotriphosphate (1000 Ci/mmoll; Dupont/NNEN, Wilmington, DE). Both strands were sequenced and analysis was performed using GeneJockey II (Biosoft, Cambridge, UK).

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LITERATURE CITED


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