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Purification and Characterization of β_2-Tomatinase, an Enzyme Involved in the Degradation of α-Tomatine and Isolation of the Gene Encoding β_2-Tomatinase from *Septoria lycopersici*

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***Lycopersicon* species often contain the toxic glycoalkaloid α-tomatine, which is proposed to protect these plants from general microbial infection. However, fungal pathogens of tomato often are tolerant to α-tomatine and detoxification of α-tomatine may be how these pathogens avoid this potential barrier. As an initial step to evaluate this possibility, we have purified to homogeneity a β-1,2-D glucosidase from the tomato pathogen *Septoria lycopersici* that hydrolyzes the β-1,2-D glucosyl bond on the tetrasaccharide moiety of α-tomatine to produce β_2-tomatine. The enzyme is a 110-kDa protein with a pI of 4.5 and a K_m for α-tomatine of 62 μM. 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 β-D-glucosidases. When *S. lycopersici* was incubated with α-tomatine, β_2-tomatinase mRNA accumulated, suggesting that the enzyme is substrate inducible. *Aspergillus nidulans* expressed "β_2-tomatinase" activity when transformed with this gene but transformants were still sensitive to α-tomatine.**

Additional keywords: glucosidase, phytoanticipin, saponins.

α-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 α-tomatine and other saponins are due to their interaction with 3β-hydroxy sterols, which results in increased membrane permeability and causes leakage of electrolytes (Keukens et al. 1992). Saponins such as α-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 Boethel 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 *Gaeumannomyces 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). *Gaeumannomyces 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 β-glucosidase that removes the β-1,2 and β-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 α-tomatine in disease resistance of tomato and for the importance of the detoxification of α-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-tomatine are more sensitive to α-tomatine 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-tomatine. Thus, it was suggested that α-tomatine may play a role in the plant's defense toward fungal saprophytes and nonpathogens of tomato and that detoxification of α-tomatine 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-tomatine in disease resistance. They induced mutants of *Nectria haematococca* that were more tolerant to α-tomatine and demonstrated that these mutants were now pathogenic on green tomato fruits, which contain high levels of α-tomatine. The wild-type strains were pathogenic only on ripe fruit, which contain little or no α-tomatine.

As a means to further evaluate the role of α-tomatine 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-tomatine 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 β_2-tomatine (Fig. 1) (Arneson and Durbin 1968). β_2-Tomatine 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 Uchytil 1969) and we report here the purification to homogeneity of this enzyme which we call "β_2-tomatinase." The nomenclature given to this enzyme, β_2-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-tomatine (Schlösser 1975; Ford et al. 1977). During the purification and characterization of β_2-tomatinase, we were informed that a partial cDNA encoding β_2-tomatinase had been cloned from a different isolate of *S. lycopersici* by its homology to the avenacinase 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 β_2-tomatinase from *S. lycopersici*, the properties of which are reported here.

RESULTS

Extraction and purification of β_2-tomatinase.

Initial attempts to purify β_2-tomatinase from culture filtrates were hampered by the secretion of melanin-like pigments when *S. lycopersici* was grown in liquid cultures. Cleavage of α-tomatine to β_2-tomatine by cell free extracts of such cultures was detected by thin layer chromatography (TLC), which verified that β_2-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 β_2-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, β_2-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 β_2-tomatinase.

β_2-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 β_2-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 β_2-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. β_2-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. β_2-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 β_2-tomatinase (Table 1).

Characterization of β_2-tomatinase activity.

β_2-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-tomatine 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 pI of 4.5.

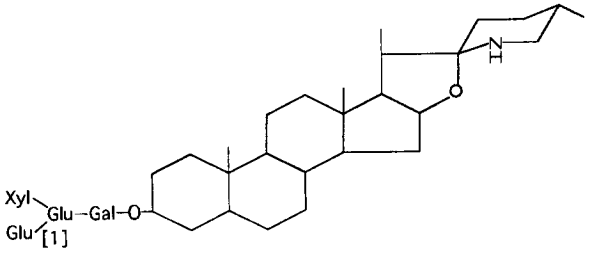

Fig. 1. Structure of α-tomatine [(O-β-D-glucopyranosyl-(1,2 glu)-O-β-D-xylopyranosyl-(1,3 glu)-O-β-D-glucopyranosyl-(1,4 gal)-β-D-galactopyranosyl-tomatidine)]. Degradation of α-tomatine to β_2-tomatine by the β_2-tomatinase enzyme of *Septoria lycopersici* (β-1,2-D glucosidase, EC 3.2.1.21) results in hydrolysis of the β-1,2-glycosidic bond at [1].

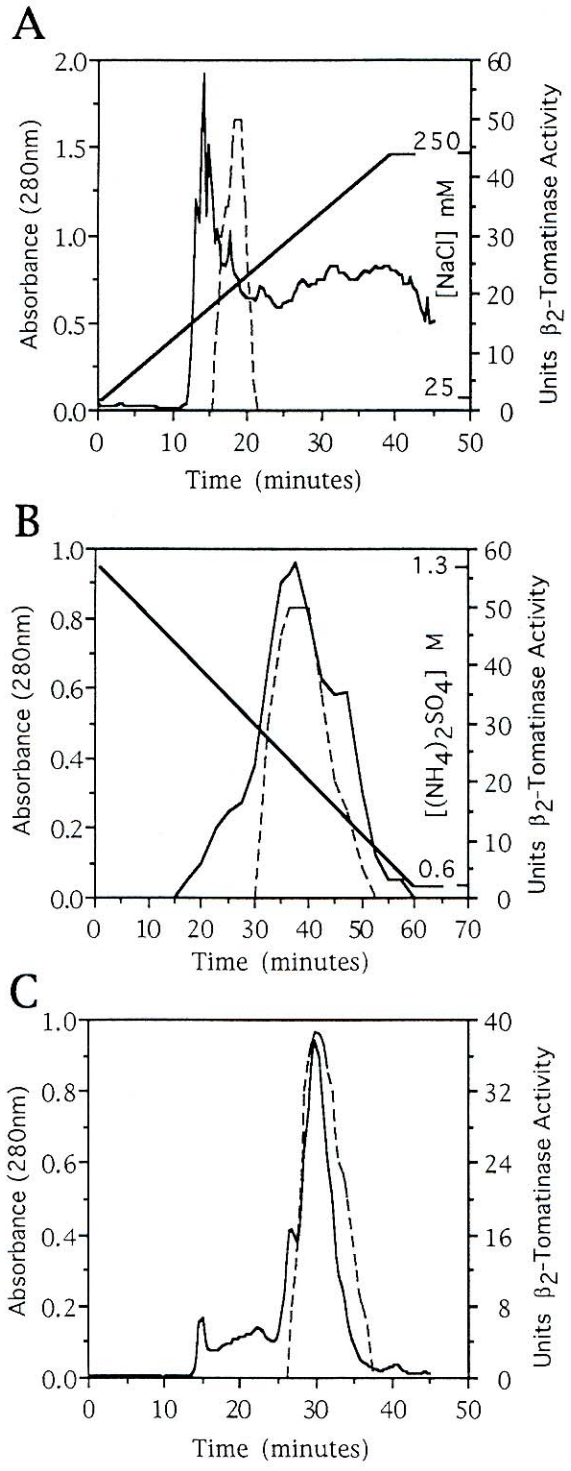

Fig. 2. Elution profiles of β_2-tomatinase from the chromatographic columns used in this study. Protein absorbance was measured at 280 nm (solid line). β_2-Tomatinase activity was determined using the alkaline ferricyanide assay (dashed line). One unit is 1 µmole glucose produced per minute at 37°C. **A,** Elution profile of β_2-tomatinase on Mono Q column. The column was eluted with a linear gradient of 25 mM-250 mM NaCl (bold solid line) in 25 mM Tris-HCl pH 7.5 at 4 ml/min. **B,** Elution profile of β_2-tomatinase on phenyl agarose column. The column was eluted with a linear gradient of 1.3 M-0.6 M ammonium sulfate (bold solid line) in 25 mM Tris-HCl pH 7.5 at 0.4 ml/min. **C,** Elution profile of β_2-tomatinase on Superose 12 column. The column was eluted with 25 mM Tris-HCl pH 7.5, 25 mM NaCl at 0.4 ml/minute.

The K_m of β_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 α-tomatine in these experiments. The enzyme had normal saturation kinetics and a V_{max} at 900 µM α-tomatine. The Hanes-Woolf method (Hanes 1932) was used to calculate K_m values; it was found that β_2-tomatinase has a K_m value of 62 µM for α-tomatine.

The substrate specificity of β_2-tomatinase was evaluated by incubating the enzyme with other saponins that had β-1,2 glycosidic linkages as well as with other simple α- or β-D glycosides and comparing the enzyme's glycosidic activity on these compounds with its activity on a similar molar concentration of α-tomatine. For these assays, the HPLC assay for released sugars was again employed in order to detect low levels of activity. β_2-Tomatinase shows a strong preference toward α-tomatine, having a relative activity of 2% on F-

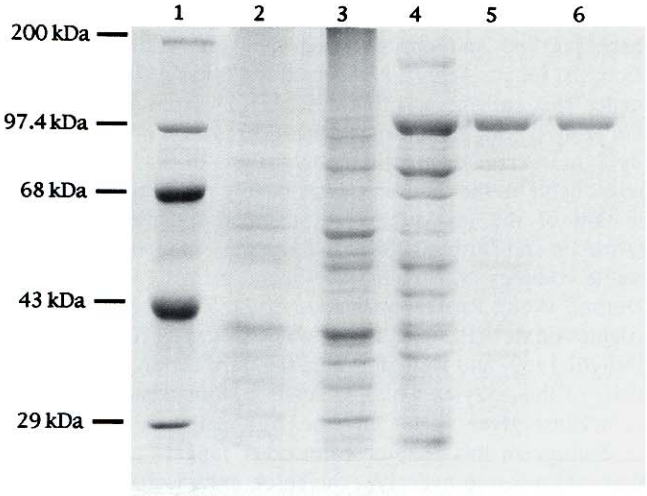

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the different preparations that had β_2-tomatinase activity during the purification of β_2-tomatinase. Lane 1, GIBCO-BRL high molecular mass markers, lane 2, 30 µg of starting mycelial protein extract; lane 3, 30 µg of protein from 50 to 80% ammonium sulfate precipitation; lane 4, 20 µg of protein with β_2-tomatinase activity from Mono Q column; lane 5, 5 µg of protein with β_2-tomatinase activity from phenyl agarose column; and lane 6, 5 µg of protein with β_2-tomatinase activity from Superose 12 column. The gel was stained with Coomassie blue R-250.

Table 1. Purification of β_2-tomatinase from *Septoria lycopersici*

Step[a]	Protein (mg)	Total activity (µmole glucose /min)	Specific activity (µmole glucose/ min/mg)	Purification (-fold)	Yield (%)
Crude extract	2,289	442	0.193	–	100
Ammonium sulfate	768	264	0.344	1.8	60
Mono Q	9.4	205	21.8	113	46
Phenyl agarose	5.0	201	40.6	210	45
Superose 12	1.3	120	92.3	478	27

[a] Samples from each chromatographic step were dialyzed in 25 mM Tris ph 7.5, 25 mM NaCl prior to activity assays.

gitonin and 0.4% on digitonin compared with its activity on α-tomatine. No activity was detected on the steroid-like or triterpenoid compounds gitonin, solasonine, and β-escin or on the glycosides stevioside, phloridzin, melezitose, cellobiose, 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 ILGGFKMEKNSQPDQ while the sequence of the 43-kDa peptide was KRTESVGGRVQYLLS. 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 β_2-tomatinase gene.

The gene encoding β_2-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 our cosmid library with this cDNA resulted in the isolation of cosmid pKBY2#6.1. A 4.1-kb *Pst*I/*Bgl*II fragment from this cosmid was subcloned into pPK1, called pβ_2TOM, and was transformed into *Aspergillus nidulans*. This clone conferred both intracellular (Fig. 4) and extracellular β_2-tomatinase activity to *A. nidulans* verifying that the β_2-tomatinase gene was present on this fragment.

Properties of the β_2-tomatinase gene.

Sequence of the 4.1-kb *Pst*I/*Bgl*II 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 β_2-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 β_2-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 β_2-tomatinase enzyme is present in the deduced open reading frame as residues 481 to 495. When the 4.1-kb *Pst*I/*Bgl*II fragment was used as a probe on a Southern blot containing *S. lycopersici* genomic DNA digested with *Pst*I a single hybridizing band of 7.0 kb was observed, which agrees with the restriction analysis of the cosmid clone pKBY2#6.1 and indicates that the β_2-tomatinase gene exists as a single copy in the genome (data not shown).

To determine whether the β_2-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 β_2-tomatinase mRNA was observed after

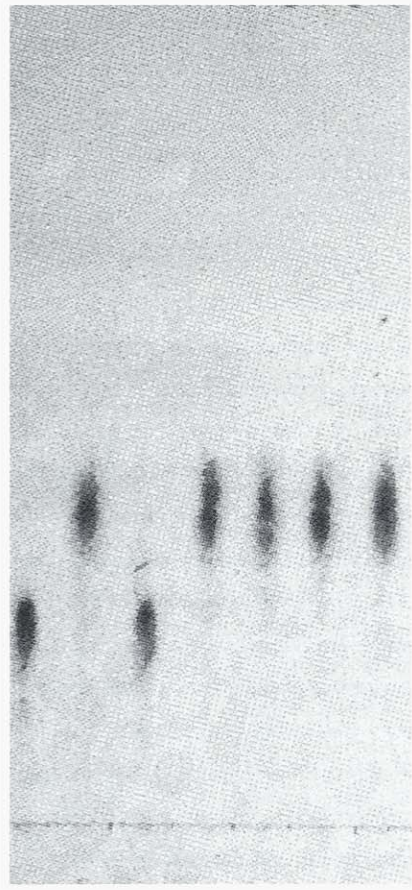

Fig. 4. Thin-layer chromatography (TLC) plate showing degradation products of *Septoria lycopersici* and transformants of *Aspergillus nidulans*. *Septoria lycopersici* β_2-tomatinase (1 μg) from the final protein purification step and crude mycelial extracts (1 mg of total protein) from *A. nidulans* UCD1 and isolates of *A. nidulans* UCD1 transformed with the *S. lycopersici* gene encoding β_2-tomatinase were added to 250 μl of 250 μM α-tomatine, 50 mM sodium acetate pH 5.0 and incubated at 37°C for 3 h. The alkaloids were precipitated by adding 25 μl of 30% ammonium hydroxide. The precipitated alkaloids were dissolved in 50 μl of ethanol. Approximately 10 μg of the alkaloids was spotted onto the TLC plate and developed in ethyl acetate, methanol, acetic acid, and water (30:20:10:1). The TLC plate was dipped in 30% sulfuric acid and charred at 110°C for 10 min. Top of figure represents solvent front. Lane 1, α-tomatine control, no enzyme (R_F = 0.23; lane 2, *S. lycopersici* reaction (β_2-tomatine R_F = 0.4); lane 3, *A. nidulans* UCD1; lane 4, *A. nidulans* transformant T1; lane 5, *A. nidulans* transformant T2; lane 6, *A. nidulans* transformant T3; lane 7, *A. nidulans* transformant T4.

2 h, remained elevated up to 8 h, and was undetectable at 24 h. This correlated with the complete hydrolysis of α-tomatine to β_2-tomatine in these cultures (Fig. 6).

A search of GENBANK with the deduced amino acid sequence of the β_2-tomatinase gene revealed that a high degree of similarity exists between this gene and several other microbial β-glucosidases. The highest degree of homology was observed with the avenacinase gene of *G. graminis* var. *avenae* (Anne Osbourn, 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*, *Saccharomycopsis 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 gilvus*, and *Ruminococcus albus* (Gräbnitz et al. 1989; Lin et al. 1990; Kashiwagi et al. 1993; Takano et al. 1992). The degree of amino acid homology between β_2-tomatinase and the other fungal β-glucosidases was 40 to 45% identical and 59 to 62% similar.

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   1  CTGCAGCAGCAGACATCCGCAGATCAACCCGACGATATGCGCCCGGATCC
  51  GAGCAGAGCACCGAGCCTACGACCATCAGAGGACGCTTGTGGCAAAGAGT
 101  TCCATAGCGTCTTACGCCCGGATACATATTACGTTGTAGCTGGGTGGGGC
 151  AGGGGCATCATCCAAGACTTGCGTTCCTCGGCCTCTACAGACTCTGTGAA
 201  GAATCATTGCAACCCCTGCTAGCGTAGGGTCCTAGACTCACTATATGGAG
 251  GAGCTGTAAACCTGGCCCTGCTCGAATAGAGAGAACCTCTTTGTTGAAGG
 301  AATTGCAATTGTGTCTTCTTGATAGTAAGAACACGGATGAGTTAGGACTC
 351  AGGAGAAAGTAACCACTCAATGTAGTTAGCATTCCACGCTAGCAGGGGTT
 401  CGAAAGATTTTTCACAGAGTCTGTATTGTGGTACGTACTCGTATTTCGTA
 451  TCGAATACGTGCGTCCTTTGCCAAACGATGGCGACAATTCGGCCGAACAT
 501  TAACGGTTGCCTAAAGGTTTAGTTAGATCCAAACGCAAGGTCCACGAGAG
 551  CGCTGTAACAAAAGCCAGTACATCCTCCAACAATTGGCATATCCAACGAG
 601  CTGCTTTACGAGACTCCGCGCGTTGACATTATAATGGTATCAAGCTTGTT
                                        M  V  S  S  L  F
 651  CAACATCGCTGCCCTTGCTGGGGCCGTGATCGCCTTGTCCCACGAAGATC
       N  I  A  A  L  A  G  A  V  I  A  L  S  H  E  D  Q
 701  AATCGAAGCACTTTACGACTATTCCGACATTCCCAACAC GTACGTGGCAG
       S  K  H  F  T  T  I  P  T  F  P  T  P
 751  ACTTGCGTGTTGCGATGCCGATACACTGACTCCCTGGGCGCAG CTGATAG
                                                 D  S
 801  CACGGGCGAAGGATGGAAGGCCGCCTTTGAGAAAGCGGCCGATGCCGTGT
       T  G  E  G  W  K  A  A  F  E  K  A  A  D  A  V  S
 851  CGCGTCTCAACCTCACGCAGAAGGTTGCGCTGACGACGGGCACGACAGCT
       R  L  N  L  T  Q  K  V  A  L  T  T  G  T  T  A
 901  GGCCTTTCCTGCAACGGAAACATCGCTCCGATTCCTGAGATCAATTTCAG
       G  L  S  C  N  G  N  I  A  P  I  P  E  I  N  F  S
 951  TGGCTTGTGTTTGGCAGATGGACCAGTATCGG GTGAGTGACACATGTTC
       G  L  C  L  A  D  G  P  V  S  V
1001  CGCGCACCGTTTGTTCACTGACTTTCGCTGTCCGGACCAG TCCGAATTGC
                                                 R  I  A
1051  AGACCTTGCCACGGTATTTCCCGCAGGCCTGACGGCTGCTGCAACTTGGG
       D  L  A  T  V  F  P  A  G  L  T  A  A  A  T  W  D
1101  ACCGCCAGCTGATCTACGAACGCGCGCGAGCACTGGGTTCTGAGTTCCGT
       R  Q  L  I  Y  E  R  A  R  A  L  G  S  E  F  R
1151  GGCAAAGGATCTCAAGTGCACCTGGG GTGAGTCTGCTTCACCCTTTCTCG
       G  K  G  S  Q  V  H  L  G
1201  GATCGATCCATTGAGAGAAGGCTGACTGGACTTCAG ACCAGCGTCTGGCG
                                              P  A  S  G  A
1251  CATTGGGTCGTCATCCGTTGGGGGGCCGCAACTGGGAGAGCTTCAGTCCT
       L  G  R  H  P  L  G  G  R  N  W  E  S  F  S  P
1301  GATCCCTACCTCAGTGGGGTGGCCATGGACTTCTCCATCCGTGGCATTCA
       D  P  Y  L  S  G  V  A  M  D  F  S  I  R  G  I  Q
1351  GGAAATGGGCGTTCAAGCGAACCGCAAGCACTTTATCGGCAATGAACAAG
       E  M  G  V  Q  A  N  R  K  H  F  I  G  N  E  Q  E
1401  AAACACAGCGCTCCAATACCTTTACGGATGACGGTACTGAGATTCAGGCA
       T  Q  R  S  N  T  F  T  D  D  G  T  E  I  Q  A
1451  ATCTCATCCAACATCGACGACCGAACCATGCACGAGTTATATCTCTGGCC
       I  S  S  N  I  D  D  R  T  M  H  E  L  Y  L  W  P
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       F  A  N  A  V  R  S  G  V  A  S  V  M  C  S  Y  N
1551  ACAGACTTAACCAGACATATGCTTGCGAGAACTCGAAGCTCATGAACGGT
       R  L  N  Q  T  Y  A  C  E  N  S  K  L  M  N  G
1601  ATTCTTAAAGGCGAGTTAGGCTTCCAGGGATATGTTGTCAGCGACTGGTA
       I  L  K  G  E  L  G  F  Q  G  Y  V  V  S  D  W  Y
1651  CGCCACCCACTCAGGAGTCGAGTCCGTCAATGCAGGTCTGGACATGACGA
       A  T  H  S  G  V  E  S  V  N  A  G  L  D  M  T  M
1701  TGCCTGGCCCTCTCGACAGTCCGTCAACCGCTTTACGACCGCCGCCATCC
       P  G  P  L  D  S  P  S  T  A  L  R  P  P  P  S
1751  TATCTTGGTGGCAACTTGACCGAAGCCGTTCTCAATGGTACTATACCCGA
       Y  L  G  G  N  L  T  E  A  V  L  N  G  T  I  P  E
1801  GGCAAGGGTCGACGACATGGCACGCCGCATCCTCATGCCATACTTTTTTC
       A  R  V  D  D  M  A  R  R  I  L  M  P  Y  F  F  L

1851  TTGGCCAAGACACGGACTTTCCAACTGTCGACCCCTCGACCGGTTTTGTC
         G  Q  D  T  D  F  P  T  V  D  P  S  T  G  F  V
1901  TTCGCTAGAACATACAACTACCCGGATGAATACTTGACACTTGGAGGCTT
       F  A  R  T  Y  N  Y  P  D  E  Y  L  T  L  G  G  L
1951  GGATCCTTACAACCCCCCGCCTGCTCGCGATGTGCGTGGTAACCATTCTG
       D  P  Y  N  P  P  P  A  R  D  V  R  G  N  H  S  D
2001  ATATTGTGCGGAAGGTTGCCGCAGCCGGGACGGTCCTTCTGAAGAACGTG
       I  V  R  K  V  A  A  A  G  T  V  L  L  K  N  V
2051  AACAACGTTCTTCCCCTCAAAGAACCCAAGTCCGTTGGTATATTCGGCAA
       N  N  V  L  P  L  K  E  P  K  S  V  G  I  F  G  N
2101  CGGTGCTGCCGATGTTACTGAAGGTCTTACATTCACCGGAGATGACAGCG
       G  A  A  D  V  T  E  G  L  T  F  T  G  D  D  S  G
2151  GACCTTGGGGTGCCGATATCGGTGCCCTTAGCGTTGGCGGAGGATCCGGT
       P  W  G  A  D  I  G  A  L  S  V  G  G  G  S  G
2201  GCCGGTAGACACACACACCTTGTTTCTCCCCTTGCAGCGATTCGCAAGCG
       A  G  R  H  T  H  L  V  S  P  L  A  A  I  R  K  R
2251  CACGGAAAGCGTGGGGGGTCGCGTCCAGTACTTGCTCAGCAACTCCAGGA
       T  E  S  V  G  G  R  V  Q  Y  L  L  S  N  S  R  I
2301  TTGTAAACGACGACTTCACATCGATCTACCCCACGCCGGAGGTTTGCCTA
       V  N  D  D  F  T  S  I  Y  P  T  P  E  V  C  L
2351  GTTTTTCTGAAAACCTGGGCTCGTGAGGGCACTGATCGTTTGTCGTACGA
       V  F  L  K  T  W  A  R  E  G  T  D  R  L  S  Y  E
2401  GAATGACTGGAACTCAACTGCCGTTGTAAACAATGTGGCTCGCCGGTGTC
       N  D  W  N  S  T  A  V  V  N  N  V  A  R  R  C  P
2451  CCAATACCATTGTCGTCACCCACTCCGGAGGCATCAACACCATGCCCCTGG
       N  T  I  V  V  T  H  S  G  G  I  N  T  M  P  W
2501  GCGGACAATGCCAATGTGACGGCGATACTCGCCGCGCACTACCCTGGTCA
       A  D  N  A  N  V  T  A  I  L  A  A  H  Y  P  G  Q
2551  GGAAAATGGGAACTCTATAATGGATATCTTGTACGGAGATGTCAATCCAT
       E  N  G  N  S  I  M  D  I  L  Y  G  D  V  N  P  S
2601  CTGGTCGCCTGCCCTACACGATACCCAAGCTGGCCACCGACTACGACTTC
       G  R  L  P  Y  T  I  P  K  L  A  T  D  Y  D  F
2651  CCGGTTGTCAACATAACTAATGAGGCTCAGGACCCATATGTTTGGCAAGC
       P  V  V  N  I  T  N  E  A  Q  D  P  Y  V  W  Q  A
2701  CGACTTCACGGAAGGTCTCTTGATCGATTACCGTCACTTTGATGCTAGGA
       D  F  T  E  G  L  L  I  D  Y  R  H  F  D  A  R  N
2751  ACATTACCCCTCTTTATGAGTTTGGCTATGGGCTCAGTTACACCACTTTC
       I  T  P  L  Y  E  F  G  Y  G  L  S  Y  T  T  F
2801  GAGATCGAGGGCGTCGCAAATCTGGTAGCAAAATCCGCCAAACTCTCCGC
       E  I  E  G  V  A  N  L  V  A  K  S  A  K  L  S  A
2851  CTTCCCTGCTTCAACGGACATCTCCCACCCCGGAGGTAATCCTGACCTGT
       F  P  A  S  T  D  I  S  H  P  G  G  N  P  D  L  W
2901  GGGAAGAGGTGGTTTCGGTAACCGCCGCAGTAAAGAACACAGGCAGCGTC
       E  E  V  V  S  V  T  A  A  V  K  N  T  G  S  V
2951  TCCGGGTCGCAGGTGGTGCAGCTGTATATTTCTCTGCCCGCGGATGGTAT
       S  G  S  Q  V  V  Q  L  Y  I  S  L  P  A  D  G  I
3001  ACCTGAGAATTCGCCAATGCAGGTTTTACGCGGCTTCGAGAAGGTTGATC
       P  E  N  S  P  M  Q  V  L  R  G  F  E  K  V  D  L
3051  TCCAACCGGGACAGTCCAAATCCGTCGAATTCTCGATTATGCGCCGCGAC
       Q  P  G  Q  S  K  S  V  E  F  S  I  M  R  R  D
3101  CTGAGTTTCTGGAACACGACGGCGCAGGACTGGGAGATTCCGAACGGCCA
       L  S  F  W  N  T  T  A  Q  D  W  E  I  P  N  G  Q
3151  GATCGAGTTCCGTGTTGGCTTTAGCTCAAGAGACATCAAGTCTATCGTTT
       I  E  F  R  V  G  F  S  S  R  D  I  K  S  I  V  S
3201  CAAGATCTTTTCTATAGAGTCCTATGTTACGGTTTGGTGCGCCGGCCCAG
       R  S  F  L  *
3251  AGGCTCGGCAGATGCCATACCGCCTCACCTCGCCCACCTCTCCCAACATA
3301  GCCGCCGAGAAGCTCTAAATAGAAGCTGCTACCTTTGCCTGGTTGTCCAC
3351  GTACTGTCCAAGCATCGCGATTCCCAACTATGGCCTTTC
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Fig. 5. Nucleotide sequence and deduced amino acid sequence of the β_2-tomatinase gene and the flanking DNA (GENBANK accession #U24701). The three introns near the 5′ end of the gene are italicized. The double-underlined region indicates the amino acid sequence obtained from the 43-kDa protease-digested peptide.

For the prokaryotic β-glucosidases there was 18 to 34% identity and 42 to 56% similarity with the β_2-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 A_3 from *Aspergillus wentii* (Bause and Legler 1980) (Fig. 7). These β-glucosidases recently have been classified as belonging to the family 3 glycosyl hydrolases based on their strong amino acid sequence similarities (Henrissat and Bairoch 1993). Although homology exists between these β-glucosidases and β_2-tomatinase it is unknown whether they possess activity toward α-tomatine.

Sensitivity of *S. lycopersici* and *A. nidulans* transformants expressing the β_2-tomatinase gene to α-tomatine and β_2-tomatine.

Septoria lycopersici, *A. nidulans,* and four transformants of *A. nidulans* that express the β_2-tomatinase gene were tested for their sensitivity toward α-tomatine and β_2-tomatine 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-tomatine and no reduction of growth on medium containing this concentration of β_2-tomatine (data not shown). The ED_{50} (effective dose whereby growth rate is reduced to 50% of that seen on control plates lacking the compound) of α-tomatine 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 β_2-tomatine, the ED_{50} 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 β_2-tomatine, the transformants were statistically more sensitive ($P = 0.05$) than untransformed *A. nidulans* at 100 μM and 150 μM β_2-tomatine (Fig. 8B).

DISCUSSION

As a first step to determine the biological role of α-tomatine degradation by the tomato pathogen *S. lycopersici,* we have purified to homogeneity the "tomatinase" enzyme that was partially purified and characterized by Durbin and Uchytil (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, β_2-tomatinase, that converts α-tomatine to β_2-tomatine and the K_m value we found for this enzyme on α-tomatine (62 μM) was similar to what they reported (60 μM). Further characterization of β_2-tomatinase using glycosides with glycosidic linkages and/or steroid or triterpenoid saponins similar to α-tomatine demonstrated that this enzyme is highly specific toward α-tomatine. Such a strong preference toward α-tomatine and the low K_m of this enzyme is consistent with the hypothesis that this tomato pathogen may have evolved a specific enzyme to detoxify this phytoanticipin.

The genomic copy of the gene encoding β_2-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 *Gaeumannomyces graminis* var. *avenae* (Bowyer et al. 1995). The avenacinase protein has an enzymatic function similar to that of β_2-tomatinase and is responsible for the detoxification of the oat phytoanticipin, avenacin. When transformed into *A. nidulans* the genomic copy of the β_2-tomatinase gene was able to confer both intracellular and extracellular β_2-tomatinase activity.

Considerable sequence homology was observed between β_2-tomatinase and other β-glucosidases belonging to the family 3 of the glycosyl hydrolases 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 β_2-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. β_2-Tomatinase activity is found in the tomato pathogens *Stemphylium solani* (R. W. Sandrock and H. D. VanEtten, unpublished), *Verticillium albo-atrum* (Pegg and Woodward 1986), and *Alternaria solani* (Schlösser 1975; R. W. Sandrock and H. D. VanEtten,

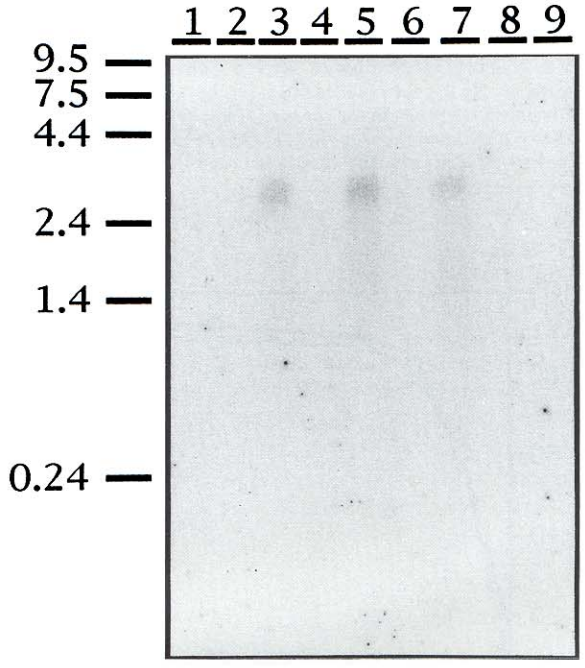

Fig. 6. Northern analysis of the induction of the *Septoria lycopersici* β_2-tomatinase gene. Total RNA was extracted from *S. lycopersici* incubated in the presence or absence of α-tomatine at various time points and 5 μg RNA from each time point was subjected to Northern analysis using the 4.1-kb *PstI*/*Bgl*II fragment containing the genomic copy of the β_2-tomatinase gene as a probe. Lane 1, 0 h; lane 2, 2 h, buffer control; lane 3, 2 h, in the presence of α-tomatine; lane 4, 4 h, buffer control; lane 5, 4 h, in the presence of α-tomatine; lane 6, 8 h, buffer control; lane 7, 8 h, in the presence of α-tomatine; lane 8, 24 h, buffer control; lane 9, 24 h, in the presence of α-tomatine.

unpublished). Other fungal pathogens of tomato, such as *Fusarium oxysporum* f. sp. *lycopersici* (Ford et al. 1977) and *Botrytis cinerea* (Verhoeff and Liem 1975), are known to produce an enzyme that cleaves the β-1-D galactosidic linkage of α-tomatine releasing the tetrasaccharide moiety of α-tomatine (refer to Figure 1). It is possible that this enzyme shares similar structural features and conserved amino acid sequences with β_2-tomatinase 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 β_2-tomatine or the release of the entire tetrasaccharide to produce tomatidine from α-tomatine were detoxification reactions because the products formed were thought to be nontoxic (Arneson and Durbin 1968b). Our results with transformants of *A. nidulans* expressing β_2-tomatinase 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 β_2-tomatinase gene in the transformants. An additional enigma is why the transformants of *A. nidulans* expressing β_2-tomatinase are more sensitive to the intermediate levels of β_2-tomatine than is the untransformed fungus (Fig. 8B). Although β_2-tomatine cannot bind to 3β-OH sterols, which are thought to be requisite for the toxic effects of α-tomatine (Arneson and Durbin 1968b), *A. nidulans* is still more sensitive to β_2-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 β_2-tomatine and α-tomatine share unknown properties that render them toxic to *A. nidulans* and some other fungi or that β_2-tomatine has a unique mode of toxicity to these fungi.

Clearly, *S. lycopersici* is highly tolerant to both α-tomatine and β_2-tomatine, even though it lacks the ability to degrade β_2-tomatine. It may be that this fungus possesses additional tolerance mechanisms to both compounds. Nondegradative tolerance mechanisms toward plant phytoalexins and polyene antibiotics that interact with sterols or membrane lipids are known to exist in several fungi (Denny and VanEtten 1983;

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Sly β2-Tom   ISSN IDDRTMHELY LWPFANAVR. SGVASVMCSY NRLNQTYACE NSKLMNGILK GELGFQGYVV SDWYATHSGV ESVNAGLDM. TMPG  302
Gga Avn      ISSN IDDRTMHEAY LWPFYNAVR. AGTTSIMCSY QRINGSYGCQ NSKTLNGLLK TELGFQGFVV SDWAATHSGV ASIEAGLDM. NMPG  301
Tre Bgl1     ISSN PDDRTLHELY TWPFADAVQ. ANVASVMCSY NKVNTTWACE DQYTLQTVLK DQLGFPGYVM TDWNAQHTTV QSANSGLDM. SMPG  289
Sfi Bgl1     ISAN IPDRAMHALY LWPFADSVR. AGVGSVMCSY NRVNNTYACE NSYMMNHLLK EELGFQGFVV SDWGAQLSGV YSAISGLDM. SMPG  317
Sfi Bgl2     ISAN IPDRAMHELY LWPFADSIR. AGVGSVMCSY NRVNNTYSCE NSYMINHLLK EELGFQGFVV SDWAAQMSGA YSAISGLDM. SMPG  322
Cpe BglS     LSSE IDDRAMHEIY LWPFAEAVR. GGVSSIMCSY NKLNGSHACQ NSYLLNYLLK EELGFQGFVM TDWGALYSGI DAANAGLDMD .MPC  320
Pca BglN     VSAN IDDKTMHELY LWPFQDSVR. AGLGSIMGSY NRVNNSYACK NSKVLNGLLK SELGFQGFVV SDWGGQHTGI ASANAGLDM. AMPS  303
Kfr BglB     SNSI VSERALREIY LEPFRLAVKH ANPVCIMTAY NKVNGDHCSQ SKKLLIDILR DEWKWDGMLM SDWFGTYTTA AAIKNGLDI. ....  249
Cth BglB     VDTI VDERTLREIY FASFENAVKK ARPWVVMCAY NKLNGEYCSE NRYLLTEVLK NEWMHDGFVV SDWGAVNDRV SGLDAGLDL. ....  242
Bfi BglA     SDSR ASERAIREIY LKAFEIIVKE QSPGASCLQY NIVNGQRSSE SHDLLTGILR DEWGFEGVVV SDWWGF.... ......GEHY KEVL  781
Cgi β-Glu    LDAR IDKAALRMSD LLAMELALEQ SDAGSVMCAY NRLNGPYTCE HPWLLSEVLK RDWGFRGYVM SDWGATHSTV AAANSGLDQQ SGQE  313
Ral β-Glu    IDSV ASERALREIY LKGFEIAVRK SKARSVMTTY GKVNGLWTAG SFDLNTMILR KQWGFDGFTM TDWWANINDR GCAPDKNNFA AMVR  718
Awe β-Glu    .... .......... .......... .......... .......... AZLGFZGFVM SDWAAHHAGV SGALAGLBMG SMPG
                                                                        *
CONSENSUS    ISS- IDDRAMHE-Y LWPF--AVR- A---SVMCSY N-VNG-Y-CE N--LL--ILK -ELGFQGFVV SDW-A---GV --A--GLDM- -MP-
```

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 = *Gaeumannomyces graminis* var. *avenae*; Tre = *Trichoderma reesii*; Sfi = *Saccharomycopsis fibuligera*; Cpe = *Candida peliculosa*; Pca = *Pichia capsulata*; Kfr = *Kluyveromyces fragilis*; Cth = *Clostridium thermocellum*; Bfi = *Butyrivibrio fibrisolvens*; Cgi = *Cellvibrio gilvus*; Ral = *Ruminococcus albus*; Awe = *Aspergillus wentii*. The asterisk indicates the aspartic acid residue of *A. wentii* β-glucosidase A_3 that binds the inhibitor conduritol B epoxide.

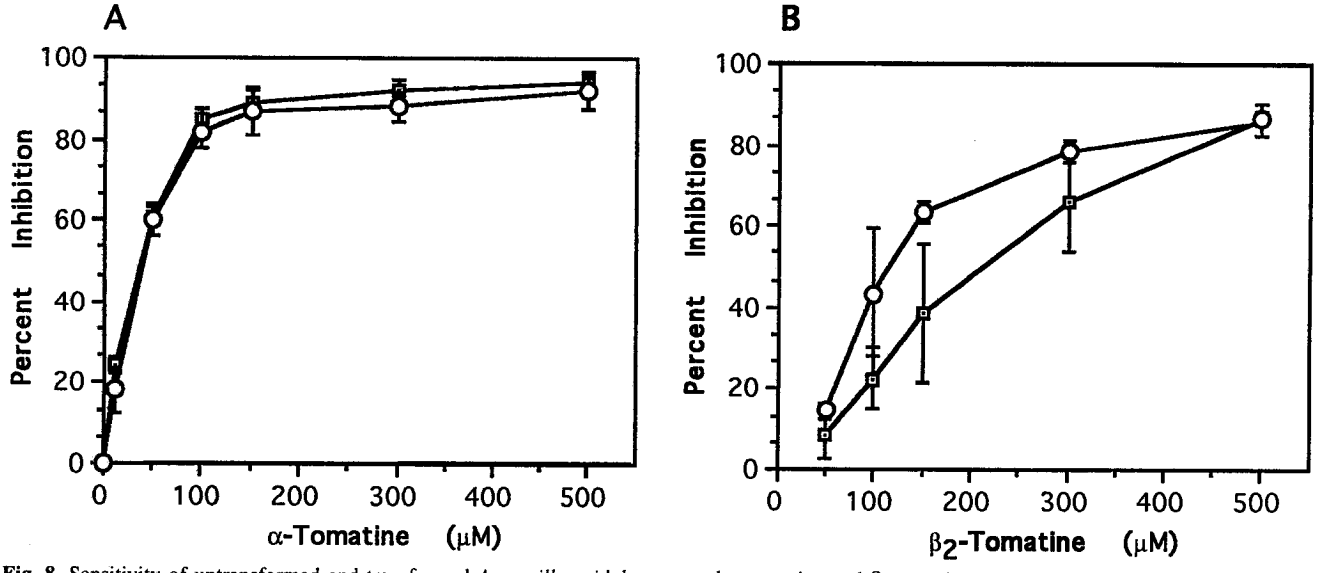

Fig. 8. Sensitivity of untransformed and transformed *Aspergillus nidulans* toward α-tomatine and β_2-tomatine. Squares represent the growth curve of untransformed *A. nidulans* while circles represent the growth curve of transformant T4 and are indicative of the growth pattern of all the *A. nidulans* 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 β_2-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 ($t_{0.05}$).

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_2-tomatinase now makes it possible to resolve some of these unknowns as it should be feasible to make β_2-tomatinase 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_2-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. Pycnidiaspores 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 $CaCO_3$, 20 g of agar per liter) for 7 days. Pycnidiaspores 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 pycnidiaspore production, 7×10^4 spores were spread onto the 150-mm V8 agar plates. Optimal production of pycnidiaspores 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 1×10^9 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 K_2HPO_4, 0.2 g of $MgSO_4$ 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_2-tomatinase 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 Arg*B 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 Uchytil (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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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_2-tomatinase 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). $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 500 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 F_{254}: 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 β_2-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 $K_3Fe(CN)_6 \cdot 3H_20$, 103 mM KCN, and 252 mM Na_2CO_3. 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 β_2-tomatinase is considered to be the amount of enzyme that catalyzes the formation of 1 µmole glucose per min at 37°C.

Characterization of β_2-tomatinase activity.

Effect of temperature on enzyme activity was determined 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 B_2-tomatinase 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). Solasonine 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, solasonine, 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 solasonine 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 β_2-tomatinase was carried out as described by Stone and Williams (1993). Briefly, 100 µg of lyophilized tomatinase was dissolved in 50 µl 8 M urea, 0.4 M NH_4HCO_3, 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 cosmid clones were isolated from the library after hybridization with the *S. lycopersici* β_2-tomatinase cDNA contained in pTOM1. A 4.1-kb *PstI*/*BglII* fragment on which the β_2-tomatinase gene resides was initially cloned from cosmid #6.1 as a 5.2-kb *PstI*/*Xba*I fragment into pBluescriptSK$^-$. This clone was then digested with *Pst*I and partially digested with *Bgl*II to obtain the 4.1-kb *PstI*/*Bgl*II fragment that contains an internal *Bgl*II site. This fragment was then subcloned into the *Pst*I and *Bam*HI sites of pBluescriptSK$^-$ and called pβ_2Tom. In order to test expression of the β_2-tomatinase gene in *A. nidulans*, the insert in pβ_2Tom was excised by digestion with *Kpn*I and *Xba*I and subcloned into the *Kpn*I and *Xba*I sites of pPK1.

To analyze the transcriptional induction of β_2-tomatinase 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 β_2-tomatinase expression in *A. nidulans*.

Cosmid clones and subclones of cosmids that hybridized to the cDNA contained in pTOM1 were screened for expression of β_2-tomatinase activity in *A. nidulans*. Cosmids and plasmids were transformed into *A. nidulans* based on the protocol developed by Yelton et al. (1983). 2×10^6 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 × *g* at 4°C. One milligram of crude protein extract in 250 µl 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 β_2-tomatine.

Septoria lycopersici and *A. nidulans* were tested for their sensitivity to α-tomatine or β_2-tomatine by radial 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 β_2-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 β_2-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 β_2-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 pBluescriptSK$^-$ and sequencing by the dideoxy chain-termination method using the M13 universal (–20) and M13 reverse sequencing primers, Sequenase (United States Biochemicals, Cleveland, OH) and deoxyadenosine 5′-α[^{35}S] thiotriphosphate (>1000 Ci/mmol; Dupont/NEN, Wilmington, DE). Both strands were sequenced and analysis was performed using GeneJockey II (Biosoft, Cambridge, UK).

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