

## Purification and Characterization of Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici*

KHALID LAIRINI,<sup>1</sup> ALONSO PEREZ-ESPINOSA,<sup>1</sup> MANUEL PINEDA,<sup>2</sup>  
AND MANUEL RUIZ-RUBIO<sup>1\*</sup>

*Departamento de Genética<sup>1</sup> and Departamento de Bioquímica y Biología Molecular,<sup>2</sup>  
Facultad de Ciencias, Universidad de Córdoba, 14071 Córdoba, Spain*

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The antifungal compound  $\alpha$ -tomatine, present in tomato plants, has been reported to provide a preformed chemical barrier against phytopathogenic fungi. *Fusarium oxysporum* f. sp. *lycopersici*, a tomato pathogen, produces an extracellular enzyme inducible by  $\alpha$ -tomatine. This enzyme, known as tomatinase, catalyzes the hydrolysis of  $\alpha$ -tomatine into its nonfungitoxic forms, tomatidine and  $\beta$ -lycotetraose. The maximal tomatinase activity in the fungal culture medium was observed after 48 h of incubation of germinated conidia at an  $\alpha$ -tomatine concentration of 20  $\mu$ g/ml. The enzymatic activity in the supernatant was concentrated against polyethylene glycol 35000, and the enzyme was then purified to electrophoretic homogeneity by a procedure that includes preparative isoelectric focusing and preparative gel electrophoresis as main steps. The purification procedure had a yield of 18%, and the protein was purified about 40-fold. Tomatinase was found to be a monomer of 50 kDa by both native gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The analytical isoelectric focusing of the native tomatinase showed at least five isoforms with pIs ranging from 4.8 to 5.8. Treatment with *N*-glycosidase F gave a single protein band of 45 kDa, indicating that the 50-kDa protein was N glycosylated. Tomatinase activity was optimum at 45 to 50°C and at pH 5.5 to 7. The enzyme was stable at acidic pH and temperatures below 50°C. The enzyme had no apparent requirement for cofactors, although  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  produced a slight stimulating effect on tomatinase activity. Kinetic experiments at 30°C gave a  $K_m$  of 1.1 mM for  $\alpha$ -tomatine and a  $V_{\max}$  of 118  $\mu$ mol/min/mg. An activation energy of 88 kJ/mol was calculated.

*Fusarium oxysporum* is a widespread soilborne plant pathogen that generally causes vascular wilts. It exists as many forms that are grouped into formae speciales on the basis of their ability to provoke disease on a particular host (1). *Fusarium oxysporum* f. sp. *lycopersici* (Sacc) Snyder & Hans. causes vascular wilt of tomato (*Lycopersicon esculentum*), resulting in severe crop losses throughout the world (4).

Plants have evolved different defense mechanisms to protect themselves against a great variety of invasive pathogens. A possible determinant of resistance of tomato plants to fungi is the presence in the plant of a preformed inhibitor of fungal growth:  $\alpha$ -tomatine (21, 25). Tomatine is a glycosidal alkaloid consisting of an aglycone moiety (tomatidine) and a tetrasaccharide moiety ( $\beta$ -lycotetraose) which is composed of two molecules of glucose and one each of galactose and xylose; the four monosaccharides form a branched structure which is attached at the C-3 position of the aglycone (Fig. 1) (21). Tomatine has been found at high concentrations (up to 1 mM) in leaves, stems, roots, and green fruit, suggesting that it may be important in resistance to potential pathogens (21). The toxic effects of tomatine are attributed to its ability to complex with membrane sterols, causing pore formation and leakage of cell contents (9, 22–24, 26). Previous studies have shown that, in general, tomato pathogens are less sensitive to tomatine than are most nonpathogenic fungi (2). Some fungi are resistant to tomatine because of their membrane composition (2, 7), while others produce specific tomatine-detoxifying enzymes (10, 12, 18, 20). These enzymes, known as tomatinases, remove either a single sugar (*Septoria lycopersici* and *Verticillium albo-atrum*

enzymes) or all four sugars (*F. oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, and *Alternaria solani* enzymes) from the steroidal  $\alpha$ -tomatine (10, 12, 18, 20, 27).

Ford et al. (12) described an inducible enzymatic activity of *F. oxysporum* f. sp. *lycopersici* which was able to detoxify  $\alpha$ -tomatine by cleaving the glycoalkaloid into the tetrasaccharide lycotetraose and tomatidine (Fig. 1). In this study, we confirmed these findings and extend them by purifying the enzyme and demonstrating that tomatinase activity from *F. oxysporum* f. sp. *lycopersici* resides in a single and inducible extracellular protein. Under native conditions, this protein shows at least five isoforms with varying pIs. The most important molecular and kinetic properties are also presented.

### MATERIALS AND METHODS

**Preparation of spore suspension.** *F. oxysporum* f. sp. *lycopersici*, isolate 42-87, race 2, was used. It was grown in potato dextrose broth medium (Difco Laboratories, Detroit, Mich.) and incubated with shaking (120 rpm) at 28°C. At 1 week following the incubation, microconidia were obtained by culture filtration through nylon cloth (Monodur; mesh size, 10  $\mu$ m). The filtrate was then centrifuged at 10,000  $\times$  g at 4°C for 15 min, and glycerol was added to the pellet at a 30% (vol/vol) final concentration. Microconidia were stored at –80°C.

**Pathogenicity test.** To confirm the formae speciales and race of the putative strain of *F. oxysporum* f. sp. *lycopersici*, greenhouse pathogenicity tests were performed with the differential tomato cultivars Moneymaker (no resistance) and Rambo (resistant to race 2). The fungal strain was grown in potato dextrose broth medium for 5 days on an orbital shaker (120 rpm) at 28°C. The culture was then filtered through nylon cloth to separate mycelia and spores. The spores were washed with sterile distilled water to obtain a final suspension of about  $5 \times 10^6$  spores per ml. Seedlings were grown for 10 days in sterile pots containing wet vermiculite until the first true leaf began to emerge. The method of inoculation was as follows. Seedlings were shaken to remove excess vermiculite, the roots of five seedlings per cultivar were dipped into the spore suspension of the isolate for 30 min, and the seedlings were then transplanted to plastic cell trays containing vermiculite with one seedling per cell. Other seedlings were dipped in water or spore suspensions of other formae speciales (*conglutinans*, *melonis*, and *niveum*)

\* Corresponding author. Phone: (34) 57-218601. Fax: (34) 57-218606.  
Electronic mail address: ge1rurum@uco.es.

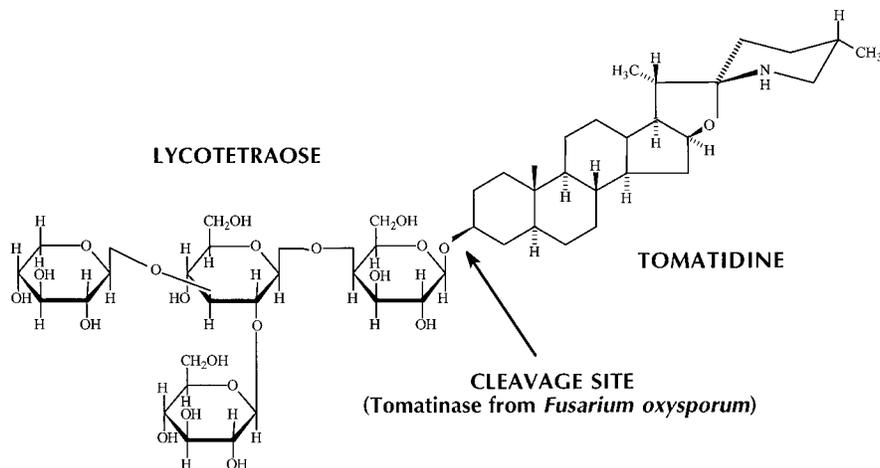


FIG. 1.  $\alpha$ -Tomatine structure and site of cleavage by tomatinase from *F. oxysporum* f. sp. *lycopersici*.

as negative controls. The susceptibility of tomato cultivars to *F. oxysporum* f. sp. *lycopersici* was recorded after 10 to 14 days.

**Fungal growth and preparation of crude extracts.** The fungal growth medium consisted of Casamino Acids (Difco) (10 g/liter), ammonium sulfate (10 mM) and yeast nitrogen base (Difco) (0.5 g/liter). Spore germination was set up by incubating the microconidia in this medium overnight with shaking (120 rpm) at 28°C.  $\alpha$ -Tomatine (Sigma) dissolved at 2 mg/ml in 50 mM potassium citrate buffer (pH 4) was added aseptically to the fungal growth medium at a final concentration of 20  $\mu$ g/ml. Culture growth was set up in 200 ml of medium containing  $2 \times 10^5$  germinated conidia per ml in a 1,000-ml Erlenmeyer flask at 28°C on gyratory shaker (120 rpm). Tomatinase activity was determined at different times of incubation. The control consisted of flasks inoculated with tomatine-free buffer. After 48 h of incubation, cultures were filtered through nylon cloth and then centrifuged at 10,000  $\times g$  for 15 min, and the supernatant was filtered through a Millipore filter (pore size, 0.22  $\mu$ m). The filtrate obtained was used directly as a crude extract to determine tomatinase activity and for the enzyme purification.

**TLC analysis of reaction products.** Crude extracts (4  $\mu$ g) from *F. oxysporum* f. sp. *lycopersici* grown in the presence (positive) and absence (negative) of tomatine were incubated overnight with 10 mM tomatine at room temperature. Incubation mixtures were vacuum dried, resuspended into an appropriate volume of methanol, and separated on Silica Gel 60 thin-layer chromatography (TLC) plates. The plates were activated at 120°C before use and were developed in a solvent system consisting of acetic acid, ethyl acetate, methanol, and water (10:30:20:1, by volume). Spots were visualized after being sprayed with 50% sulfuric acid and heated at 110°C. The metabolites were identified by cochromatographic analysis with standards (tomatine and tomatidine, both 10 mM), which were treated as incubation mixtures. Tomatine appeared as a black spot near to the origin, while tomatidine was localized as a dark-green spot near the solvent front.

**Tomatinase activity assay.** Tomatinase activity was assayed spectrophotometrically at 520 nm with the dinitrosalicylic acid reagent for determination of reducing sugars (3, 17). Unless otherwise specified, the standard assay mixture contained, in a final volume of 1 ml, 20 mM sodium acetate buffer (pH 5.5), 1 mM tomatine, and an adequate amount of enzyme preparation. The reaction was carried out at 30°C. At different reaction times (0 to 30 min), the amount of  $\beta$ -lycotetraose formed from  $\alpha$ -tomatine was measured in 0.2-ml aliquots of reaction mixture after the hydrolysis was stopped by adding 0.2 ml of 3,5-dinitrosalicylic acid solution and boiling for 5 min (17). Then 0.6 ml of water was added, and the  $A_{520}$  was recorded. One unit of enzyme activity is defined as the amount of enzyme that forms 1  $\mu$ mol of  $\beta$ -lycotetraose per min under optimal assay conditions. Specific activity is expressed in units per milligram of protein.

**Tomatinase purification. (i) PEG concentration of the sample.** The 200 ml of culture filtrate was transferred into dialysis tubing (10,000-Da cutoff) and concentrated approximately 10-fold by placing the tubing in solid polyethylene glycol (PEG) 35000 (8). When the filtrate was sufficiently concentrated, the remaining PEG was removed by thoroughly washing the dialysis membranes with distilled water. The PEG-concentrated sample was then dialyzed against distilled water overnight at 4°C with gentle shaking.

**(ii) Preparative isoelectric focusing.** The above sample (55 ml) containing 2.8 mg of total protein was loaded into a Rotofor cell (Bio-Rad Laboratories) for initial fractionation in a wide-range pH gradient (pH 3 to 10) buffered with 2% Bio-Rad Bio-Lyte ampholytes mixtures. Constant power (12 W) was applied for 4 to 5 h with the system cooled to 4°C. Runs were terminated when the voltage had stabilized for about 30 min. Twenty Rotofor fractions of 2.8 ml were col-

lected, and those with tomatinase activity were pooled and refractionated in the Rotofor cell without additional ampholytes. Upon refractionation, the positive tomatinase activity fractions were first brought to 1 M salt and then dialyzed against water overnight at 4°C to remove the ampholytes bound to proteins. The resulting fraction was PEG concentrated as above and then subjected to preparative gel electrophoresis.

**(iii) Preparative gel electrophoresis.** The discontinuous native polyacrylamide gel electrophoresis (PAGE) of the Ornstein-Davis system was used as described in the model 491 Prep Cell instruction manual (Bio-Rad Laboratories). The acrylamide concentration of the separating gel was optimized at 6%. The gel length was 5 cm. The sample in buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 0.025% bromophenol blue) was loaded onto the Prep Cell, and the gel was run for 8 h. Running buffer (25 mM Tris, 192 mM glycine [pH 8.3]) was pumped through the elution chamber at a rate of 0.5 ml/min. One hundred fractions of 2.5 ml each were collected and assayed for tomatinase activity. Positive fractions were analyzed by sodium dodecyl sulfate (SDS)-PAGE to visualize their degree of purity. Fractions containing the purest tomatinase were pooled, PEG concentrated, and used as the source of pure enzyme.

**Molecular weight determination. (i) Denatured protein (SDS-PAGE).** The molecular size was determined by SDS-PAGE (16). Enzyme preparation was boiled for 5 min in the presence of 1.6% (wt/vol) SDS and 4% (vol/vol)  $\beta$ -mercaptoethanol, with 0.1% bromophenol blue as the tracking dye. Samples were then subjected to SDS-PAGE in 10% polyacrylamide gels. The SDS-PAGE was also performed in the absence of  $\beta$ -mercaptoethanol. The following standards were used as markers: rabbit muscle myosin (205 kDa), *Escherichia coli*  $\beta$ -galactosidase (116 kDa), phosphorylase *b* from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and erythrocyte carbonic anhydrase (29 kDa). Electrophoresis was performed at a constant current of 20 mA per gel, using a vertical system (Mini Gel system; Bio-Rad Laboratories). Gels were silver stained at room temperature by the method of Heukeshoven and Dernick (15).

**(ii) Native PAGE.** The molecular size under native conditions was estimated by relative mobilities in different acrylamide concentrations (7.5, 8.5, and 10%) by the method of Hedrich and Smith (14). The following standards were used as markers: bovine serum albumin (66 and 132 kDa [monomer and dimer, respectively]), ovalbumin (45 kDa), and erythrocyte carbonic anhydrase (29 kDa). Electrophoresis was performed as described for SDS-PAGE.

**Isoelectric point determination.** Analytical isoelectric focusing of the native pure protein was performed as described in protocols supplied with the PhastSystem apparatus (Pharmacia Biotech, Inc.). Isoelectric focusing gels had a range of pH 3 to 9. The gels were stained with silver nitrate as described in the PhastSystem manual. Isoelectric focusing markers were from Pharmacia. pIs of tomatinase isoforms were estimated from a regression equation of the standard proteins versus distance migrated.

**Protein deglycosylation.** N-linked carbohydrates were removed from the pure tomatinase with *N*-glycosidase F (Boehringer, Mannheim, Germany) as specified by the manufacturer.

**Protein determination.** The protein concentration was determined as described by Bradford (6) with bovine serum albumin as standard.

**pH stability of tomatinase.** Tomatinase was preincubated overnight at 4°C in 20- $\mu$ l buffer solutions of 10 mM citric acid and 10 mM Tris (1:1) adjusted to pH 2.5 to 9.5 with HCl or NaOH. Activity was determined by adding 180  $\mu$ l of tomatine in 20 mM sodium acetate buffer (pH 5.5) at final concentration of 1 mM and incubating the preparation for 30 min at 30°C.

**Optimum pH.** The activity of purified tomatinase was measured in the same

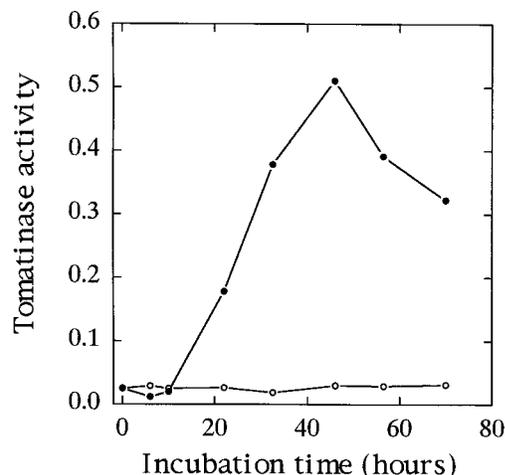


FIG. 2. Kinetics of tomatinase induction. Tomatine dissolved in 50 mM citrate buffer (pH 4) was added to 20 ml of fungal medium containing  $2 \times 10^5$  germinated conidia in a 100-ml flask. The final tomatine concentration was 20  $\mu\text{g/ml}$  (●). The control consisted of flasks inoculated with tomatine-free buffer (○). Tomatinase activity is expressed as the  $A_{520}$  of reaction mixtures consisting of 100  $\mu\text{l}$  of crude extract (taken at the times indicated) in a final volume of 0.2 ml (20 mM sodium acetate [pH 5.5], 1 mM tomatine) after overnight incubations at 30°C.

Tris-citrate buffer solutions of different pHs as described above. The enzyme was added to initiate the reaction, and tomatinase activity was determined after 30 min of reaction at 30°C.

**Temperature stability.** Enzyme preparations were incubated at 20, 30, 40, 50, 60, and 70°C. For each temperature treatment, samples were removed after 30 min, 1 h, and 2 h and their tomatinase activity was assayed as described above.

**Optimum temperature.** The tomatinase activity of the purified enzyme was determined after 10 min of reaction at 20, 30, 37, 45, 50, or 60°C and optimum pH.

**Effect of metals.** An enzyme preparation obtained after PEG concentration and preparative isoelectric focusing separation was incubated at room temperature in the presence of 0.1 mM each  $\text{MnSO}_4$ ,  $\text{CoCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{MgCl}_2$ ,  $\text{ZnSO}_4$ ,  $\text{FeCl}_3$ ,  $\text{CaCl}_2$ , or  $\text{NiSO}_4$  for 2 h. Tomatine was then added, and tomatinase activity was tested after 30 min of reaction at 37°C. The assay was carried out in 20 mM sodium acetate buffer (pH 5.5). The measured activities were compared with the activity of the enzyme solution without metal salt. As a control, the effect of these metals on the reduction of 3,5-dinitrosalicylic acid solution by glucose was assayed. Tomatinase activity was also assayed in the presence of 1 mM EDTA.

**Kinetic parameters of tomatinase.** The reaction mixture contained the substrate ( $\alpha$ -tomatine) at 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, and 10 mM dissolved in 20 mM sodium acetate buffer (pH 5.5). The reaction was started by addition of tomatinase, and the hydrolysis of the substrate was determined spectrophotometrically. The kinetic constants  $K_m$  and  $V_{max}$  were calculated from the double-reciprocal plots.

**Preparation of protein for sequence determination.** SDS-PAGE (10% polyacrylamide) of the 50-kDa protein preparation was performed. After electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine [pH 8 to 8.5], 20% methanol) for 5 min. During this time, an Immobilon-P membrane was rinsed with 100% methanol and stored in transfer buffer. The gel and Immobilon membrane were sandwiched between 3MM paper and assembled into the blotting cassette (Mini Trans-Blot electrophoretic transfer cell; Bio-Rad Laboratories). The transfer was carried out at a constant 0.4 A for 75 min. The Immobilon membrane was then rinsed three times (5 min each) in Milli-Q water, stained with Coomassie blue R-250 (0.1% in 50% methanol) for 5 min, and then destained in 50% methanol-10% acetic acid at room temperature. The membrane was finally rinsed with several changes of water, air dried, and stored at -20°C. The stained band containing tomatinase was used to analyze the N-terminal amino acid sequence.

**Amino acid sequence analysis.** N-terminal sequencing of purified tomatinase was performed at the protein-sequencing facilities of the Universitat Autònoma de Barcelona, Bellaterra Barcelona, Spain, on a model LF3000 sequencer (System Gold; Beckman).

## RESULTS

**Pathogenicity tests.** The formae speciales and race identity of *F. oxysporum* f. sp. *lycopersici*, race 2, isolate 42-87 were

confirmed in the pathogenicity test, in which all of the susceptible seedlings inoculated with this organism showed symptoms 10 to 14 days after inoculation whereas no symptoms were observed on seedlings of the resistant cultivar. Moreover, none of the other formae speciales used as controls induced wilt in the tomato seedlings.

**Induction and identification of tomatinase.** Figure 2 shows the induction kinetics of tomatinase activity in fungal culture containing  $\alpha$ -tomatine. No tomatinase activity was detected in the absence of  $\alpha$ -tomatine in the culture medium; however, a remarkable increase in the enzyme activity was observed after 10 h in the presence of the glycoalkaloid, the maximal tomatinase activity being observed after 48 h of incubation. Higher concentrations of  $\alpha$ -tomatine in the culture medium did not substantially improve the peak of maximal tomatinase activity (data not shown).

TLC analysis showed that the positive crude extract converted the glycoalkaloid  $\alpha$ -tomatine into two products, only one of which was visible on a TLC plate as a fast-moving spot corresponding to the tomatidine form (Fig. 3). However, no degradation of the  $\alpha$ -tomatine was observed when the crude extract from *F. oxysporum* f. sp. *lycopersici* grown in the absence of  $\alpha$ -tomatine was used as the source of enzyme (Fig. 3).

**Tomatinase localization in acrylamide gels.** The enzyme was detected by comparing the SDS-PAGE profile of the extracellular total protein of the filtrate from a positive culture (fungal growth in the presence of  $\alpha$ -tomatine) with that of a negative one (fungal growth in the absence of  $\alpha$ -tomatine). Figure 4 (lanes 2 and 3) shows the presence in the positive culture of a protein of about 50 kDa that is absent in negative control (lane 1). This finding was confirmed by preparing two native gels (7.5% acrylamide) and silver stained one of them. To determine enzyme activity, the nonstained gel was cut in slices of 0.5 cm each from the top. The gel slices were first frozen in liquid nitrogen and then macerated by the method of Hames (13). Proteins were collected from the gel into 2 ml of Milli-Q water. Gel fragments were removed by centrifugation, and the tomatinase activity in the supernatant was determined spectrophotometrically as described in Materials and Methods. The extracts from different slices were also subjected to SDS-PAGE (10% acrylamide). The results (Fig. 4, lanes 4 to 6) showed that



FIG. 3. TLC analysis of tomatinase reaction products. Tomatine (10 mM) was incubated overnight at room temperature with 4  $\mu\text{g}$  of either a positive (lane 2) or negative (lane 3) crude extract. Lanes 1 and 4 are controls containing  $\alpha$ -tomatine (10 mM) and tomatidine (10 mM), respectively.

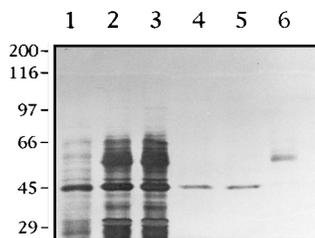


FIG. 4. SDS-PAGE (10% polyacrylamide) for tomatinase detection in crude extracts and in fractions eluted by native PAGE (7.5% polyacrylamide). Lanes: 1, negative control consisting of crude extract from *F. oxysporum* f. sp. *lycopersici* grown in the absence of tomatine; 2 and 3, positive controls containing crude extract from *F. oxysporum* f. sp. *lycopersici* grown in the presence of tomatine; 4 and 5, proteins from slices cut 0.5 and 1 cm, respectively, from the top of the native PAGE gel (these samples did not show significant tomatinase activity); 6, proteins from a slice cut 1.5 cm from the top of the native PAGE gel (this sample showed tomatinase activity).

tomatinase activity was associated exclusively with the inducible 50-kDa protein, thus ruling out the possibility of the existence of other extracellular proteins with tomatinase activity.

**Purification of tomatinase.** Tomatinase from *F. oxysporum* f. sp. *lycopersici* was purified to electrophoretic homogeneity by the steps listed in Table 1. After the initial cleanup of the crude sample, the preparative isoelectric focusing and the preparative gel electrophoresis were highly efficient in purifying the tomatinase to homogeneity. Positive fractions from the first Rotofor cell run were pooled and refocused. Fractions with the highest tomatinase activity after the second preparative isoelectric focusing were pooled, concentrated with PEG, and subjected to preparative gel electrophoresis by native PAGE of the Ornstein-Davis system. After this final purification step, tomatinase activity was observed in fractions 10 to 40 (100 fractions of 2.5 ml were collected), with the highest tomatinase purity being found in fractions 15 to 25 as observed by SDS-PAGE (result not shown). The enzyme was purified 40-fold with a 18% yield. The fact that a pure enzyme was obtained even with such a low purification factor indicates that the enzyme was highly expressed under the culture conditions used (Fig. 4). Under the routine assay conditions, the final enzyme preparation had a specific activity of 60 units mg of protein<sup>-1</sup>. The specific activity at saturating substrate concentrations ( $V_{max}$ ) is 118 units mg of protein<sup>-1</sup> (see below).

**Molecular and catalytic properties.** The molecular mass of the purified enzyme, as determined by SDS-PAGE, was estimated to be 50.4 kDa (Fig. 5A). Samples treated with and without  $\beta$ -mercaptoethanol showed a single band with identical molecular mass (results not shown), thus indicating the absence of interchain disulfide bridges. The molecular mass of the purified protein under nondenaturing conditions was also about 50 kDa, thus showing that the protein did not contain subunits. However, this native protein exhibited at least five

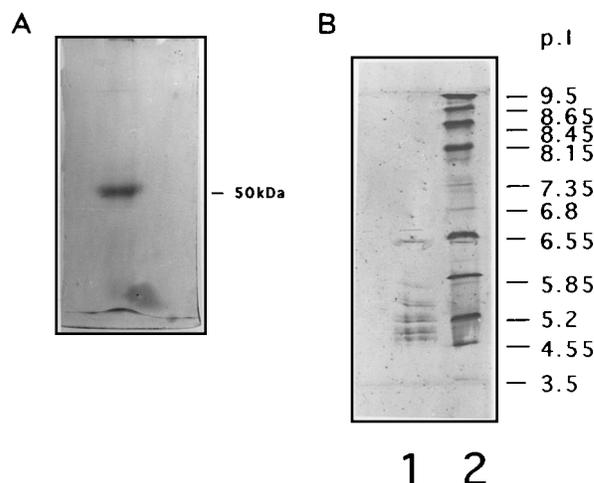


FIG. 5. (A) SDS-PAGE of the purified tomatinase (0.8  $\mu$ g). (B) Analytical isoelectric focusing of the native purified tomatinase. Lanes: 1, different isoforms of the native tomatinase (the two closely spaced bands corresponding to pI 6.6 are due to marks of the PhastSystem applicator); 2, isoelectric focusing markers. Gels were silver stained.

isoforms with pIs of 4.8, 4.9, 5.2, 5.4, and 5.8, as shown by analytical isoelectric focusing gel (Fig. 5B).

Treatment of tomatinase with *N*-glycosidase F (which hydrolyzes all types of asparagine-linked oligosaccharides) gave a product with a molecular mass of 45 kDa (Fig. 6), indicating that the 50-kDa protein was glycosylated.

Tomatinase exhibited optimum activity at pH 5.5 to 7, with a rapid decline of activity at higher and lower pHs. The tomatinase was stable in the pH range of 3 to 7 but was less stable in the alkaline range, with 40% loss of activity at pH 9.5. The enzyme was considerably more stable at temperatures under 50°C. At 60°C, it lost 40% of its activity in 30 min, and at 70°C, it was completely inactivated in the same time. The optimum temperature for enzyme activity was between 45 and 50°C. An activation energy of 88 kJ mol<sup>-1</sup> was calculated from the Arrhenius equation.

Co<sup>2+</sup> and Mn<sup>2+</sup> were found to have a slightly stimulating effect (about 20%) on the activity of tomatinase. No effect on activity was detected with Fe<sup>3+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, or Ca<sup>2+</sup>. On the other hand, the addition of EDTA to the enzyme did not affect its activity, suggesting that no metals are needed for the enzymatic reaction.

The activity of tomatinase was measured by the hydrolysis of  $\alpha$ -tomatine into tomatidine and lycotetraose, as observed in TLC analysis (Fig. 3). Under standard assay conditions, the reaction was linear with time for at least 30 min and proportional to the enzyme concentration. Tomatinase exhibited typical saturable kinetics, and experiments at 30°C with different

TABLE 1. Purification of tomatinase from *F. oxysporum* f. sp. *lycopersici*<sup>a</sup>

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Recovery (%)	Purification factor
Crude	4.3	6.5	1.5	100	1
PEG	2.8	5.3	1.9	82	1.2
Preparative isoelectric focusing (2nd run)	0.4	4.2	10.5	65	7
Preparative gel electrophoresis	0.02	1.2	60	18	40

<sup>a</sup> The purification was carried out with 200 ml of culture filtrate.

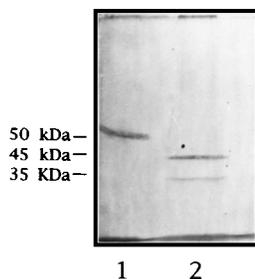


FIG. 6. SDS-PAGE of pure tomatinase after deglycosylation. Lanes: 1, pure tomatinase present as a single band of 50 kDa; 2, deglycosylated pure tomatinase. The upper band in lane 2 (45 kDa) corresponds to the deglycosylated tomatinase, and the lower one (34.6 kDa) corresponds to the *N*-glycosidase F enzyme.

concentrations of substrate gave a  $K_m$  of 1.1 mM and a  $V_{max}$  of 118  $\mu\text{mol}/\text{min}/\text{mg}$  of protein. The regression coefficient of the double-reciprocal plot was 0.9997. From these data, a turnover number (molecular weight, 50,000) of  $6 \times 10^3 \text{ min}^{-1}$  can be deduced for the enzyme working under optimal conditions.

The first seven amino acids from the N-terminal region of tomatinase were determined. The sequence obtained (Val, Lys, Ile, Pro, His, Thr, Gly) did not show significant homology with the N-terminal region of any other glycosyl hydrolase when compared with the SwissProt library by using the FASTA program.

## DISCUSSION

The production by *F. oxysporum* f. sp. *lycopersici* of an inducible extracellular enzyme that detoxifies  $\alpha$ -tomatine, previously reported by Ford et al. (12), has been confirmed. This enzyme was absent when there was no tomatine in the culture medium and increased in the presence of  $\alpha$ -tomatine (Fig. 2). The increase in tomatinase activity, or tomatinase induction, seems to be due exclusively to the presence of tomatine in the medium. Tomatine breakdown products (tomatidine and sugars) were tested for their ability to induce tomatinase activity, and none were observed to be tomatinase inducers (16a). Inducible tomatinase activities have been described for *V. albo-atrum*, *B. cinerea*, and *A. solani*, whereas *S. lycopersici* produces a constitutive tomatinase (10, 12, 18, 20, 27).

The inducibility property of tomatinase from *F. oxysporum* f. sp. *lycopersici* was used to localize and purify the enzyme. We detected a unique protein species with tomatinase activity in extracellular filtrates under the *in vitro* conditions used in the laboratory. This enzyme has been purified for the first time to electrophoretic homogeneity through a three-step procedure involving concentration with PEG, preparative isoelectric focusing, and preparative gel electrophoresis. By this procedure, we harvested an enzyme with a specific activity of 60 U/mg of protein (118 U/mg under saturating substrate concentrations), which is much higher than that reported for the partially purified tomatinase from *S. lycopersici* (10). Tomatinase purification was first attempted by precipitation with acetone (2.5 volumes) followed by gel filtration and ion-exchange chromatography, but with a such procedure only a partial purification of the enzyme was achieved (results not shown).

We found that native tomatinase from *F. oxysporum* f. sp. *lycopersici* is a monomer of about 50 kDa. This result contrasts with the molecular mass reported for tomatinase from *S. lycopersici* and avenacinase (which also has a weak tomatinase activity) from *Gaeumannomyces graminis*. Both enzymes possess a molecular mass of about 116 kDa as determined by

SDS-PAGE (18, 19). The action of the tomatinase from *S. lycopersici* is mechanistically similar to that of avenacinase from *G. graminis* and to that of the tomatinase from *V. albo-atrum* and involves the removal of a single sugar, the terminal  $\beta$ 1,2-linked glucose, from  $\alpha$ -tomatine (10, 20). In contrast, tomatinase from *F. oxysporum* f. sp. *lycopersici* removes all four sugars, cleaving the  $\beta$ 1-linked galactose and releasing the tetrasaccharide lycotetraose and tomatidine (Fig. 3), in agreement with the results reported by Ford et al. (12). Such a mode of action of tomatinase from *F. oxysporum* f. sp. *lycopersici* is similar to that reported for the tomatinase from *Botrytis cinerea* (27). The tomato pathogen *A. solani* also degrades  $\alpha$ -tomatine to tomatidine but does so by a release of monosaccharides rather than of a tetrasaccharide (25). Deglycosylation may be sufficient in all cases to destroy the ability of the tomatine to complex with membrane sterols and therefore to eliminate its toxic effect (2).

After the preparative isoelectric focusing, tomatinase activity was observed in fractions with a pH range of 4.8 to 6.0, the maximum being found in a fraction with a pH around 5.5. The broad peaks of activity observed in both preparative isoelectric focusing and preparative gel electrophoresis are presumably not surprising, given that tomatinase under native conditions shows at least five isoforms with pIs ranging from 4.8 to 5.8 (Fig. 5). Such isoforms have the same molecular mass of 50 kDa as shown in SDS-PAGE (Fig. 5). Pure tomatinase treated with *N*-glycosidase F also gave a single band of 45 kDa, indicating the glycosylated states of tomatinase isoforms (Fig. 6). This result suggests that there is only one gene encoding tomatinase; after transcription and translation, the protein probably suffers posttranslational modifications, resulting in the isoforms. This supposition is sustained by the fact that no evidence for the presence of more than one protein species in the amino acid sequence data was detected when the 50-kDa protein was sequenced. The presence of glycosylated extracellular enzymes has been described frequently, and it has been reported that glycosylation aids protein folding, stabilizes protein conformation, confers resistance to proteolytic degradation, and facilitates protein transport (11). We found an optimum pH range of tomatinase activity from *F. oxysporum* f. sp. *lycopersici* between pH 5.5 and 7. This result may be related to the fact that tomatine reacts optimally with membrane sterols at pH 7 (23). Thus, when the cellular environmental pH is around 7, the enzyme has maximal activity by avoiding membrane disruption. The optimum pH for tomatinase activity from *S. lycopersici* is 5.8 (10). Similarly, tomatinases from *S. lycopersici* (10) and *F. oxysporum* f. sp. *lycopersici* are stable up to 50°C and rapid inactivation takes place above 60°C.

The *in vivo* function of tomatinases in pathogenesis remains unknown. However, the high concentration of tomatine found in tomato plants and its probable involvement in resistance suggest a possible role of these enzymes in pathogenicity. Recently, Bowyer et al. have reported that avenacinase from the oat pathogen *G. graminis* var. *avenae*, which detoxifies avenacin, is essential for pathogenicity (5). Avenacinase deglycosylates avenacin, a fungitoxic compound related to tomatine present in oats. The purified tomatinase from *F. oxysporum* f. sp. *lycopersici* will enable us to use the information from amino acid sequence to clone the gene encoding tomatinase, a possibility which is presently under investigation in our laboratory. Furthermore, the cloned gene will allow us to investigate the role of tomatinase in the pathogenicity of *F. oxysporum* f. sp. *lycopersici* by gene disruption.

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