weight[5,6] and is in marked contrast to the complicated subunit structure of the DPN-linked enzyme[12].

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Purification and properties of a fungal β-glucosidase acting on α-tomatine

Crude mycelial extracts or culture filtrates from the fungus *Septoria lycopersici* convert α-tomatine, a steroidal glycoalkaloid, to β_2-tomatine[1]. This removal of a β-1,2-linked glycosyl unit markedly reduces fungitoxicity. Since *S. lycopersici* is parasitic on tomato leaves, organs which normally contain between 0.8 and 2.5% α-tomatine on a dry weight basis[2], we were interested in studying the reaction in more detail and determining whether it has any significance *in vivo*. This paper describes (1) a partial purification of the enzyme responsible for hydrolysis of α-tomatine and (2) some of the properties of the enzyme.

Enzyme activity was assayed spectrophotometrically at 460 nm in a reaction mixture containing 10 mM sodium phosphate buffer (pH 5.6), 10 units of glucose oxidase (Worthington GOP), 2 units of horseradish peroxidase (Worthington HPOD), 500 μM *o*-dianisidine, 250 μM α-tomatine and 0.2 unit or less of enzyme in a total volume of 2.0 ml. A unit of enzyme activity is defined as the amount of enzyme that would catalyze the formation of 1 μmole of glucose per min at 40°. Protein was determined by the method of Lowry *et al.*[3].

We inoculated flasks containing Czapek's medium supplemented with acid-hydrolyzed casein (10 g/l) and thiamine·HCl (10 mg/l) with a conidiospore suspension of *S. lycopersici*. The cultures were then incubated on a circular shaker at 24° for 8 days. The mycelium (approx. 50 g wet wt./l medium), after washing in cold 50 mM sodium phosphate buffer (pH 5.6), was suspended in the same buffer (1:2, w/v) at 0° and ground in a Duall tissue homogenizer. The extract was centrifuged (30 000 × *g* for 15 min), and the turbid supernatant fraction filtered through Whatman No. 1 filter paper. The cell-free supernatant was then treated with solid $(NH_4)_2SO_4$ without further pH control to give 50% satn. After stirring for 30 min at 0° the suspension was centrifuged (35 000 × *g* for 15 min), and the supernatant portion was treated with additional $(NH_4)_2SO_4$ to give a final concentration of 70%. After centrifugation (35 000 × *g* for 15 min) the pellet was dissolved in buffer and dialyzed for 5 h against distilled water.

The dialyzed protein fraction was applied to a column of DEAE-Sephadex A-50 (1.5 cm × 32 cm) equilibrated with 10 mM sodium phosphate buffer (pH 5.6) and a linear NaCl gradient (0.1–0.5 M in 500 ml) used for elution. The fractions containing the enzyme (0.29–0.35 M NaCl) were pooled and concentrated by using a second 70% $(NH_4)_2SO_4$ precipitation step. After centrifugation (35 000 × *g* for 15 min) the pellet was dissolved in 10 mM sodium phosphate buffer (pH 5.6). This solution was applied to a column of Sephadex G-200 (1.5 cm × 32 cm) equilibrated with the same buffer. Development was performed with 10 mM sodium phosphate buffer (pH 5.6). The enzyme emerged with the high-molecular-weight protein fraction.

The results of a representative purification of the enzyme are presented in Table I. The enzymatic activity recovered during purification was 26% and the specific activity gain was approx. 100-fold. No loss in enzyme activity occurred when the purified enzyme preparation was stored at 3° for 6 months. This purified preparation hydrolyzed α-tomatine and α-demissin. No reaction was detected with β_1-tomatine, sophorose, β-D-1,2-glucan from *Agrobacterium tumefaciens* (1 mg), laminarindextrins (1 mg), cellobiose, β-gentiobiose, β-phenyl-D-glucopyranoside and *p*-nitrophenyl-β-D-glucopyranoside using the standard assay conditions. The apparent K_m fo the enzyme for α-tomatine was 0.06 mM. The optimum pH for activity is 5.8; above this value the free base of α-tomatine begins to precipitate. Preincubation of the enzyme at 50° for 10 min did not affect activity. However, at 60° more than 90% of the enzymatic activity was lost during the 10-min preincubation period. The culture filtrate also contains an enzyme having the same activity. However, because the filtrate contained substances which interfered with purification, it has not been studied extensively.

TABLE I

PURIFICATION OF A β-1,2-GLUCOSIDASE FROM *S. lycopersici*

Fraction	*Total vol. (ml)*	*Protein (mg/ml)*	*Total enzyme units*	*Specific activity (units/mg)*	*Recovery (%)*
1. Crude extract	500	3.1	155	0.1	—
2. 50–70% satd. $(NH_4)_2SO_4$	25	1.5	75	2	48
3. DEAE-Sephadex	24	0.3	54	7.5	35
4. Sephadex G-200	20	0.2	40	10	26

To further characterize the enzyme we examined some of the criteria established by REESE *et al.*[4] to differentiate between exoglucanases and glucosidases. Enzyme activity was not inhibited by methyl-β-D-glucopyranoside at concentrations up to 10 times that of α-tomatine; however, δ-gluconolactone reduced activity about 50% at a concentration equal to that of the substrate. The anomeric form of the glucose produced during hydrolysis was also determined, the reaction being carried out at 0° in the absence of buffer to minimize mutarotation. At intervals aliquots were withdrawn and lyophilized, then the trimethylsilyl derivative was prepared and subjected to gas–liquid chromatography[5]. The results showed that there was a retention of the β configuration in the glucose released.

Judging from these results the enzyme which hydrolyzes α-tomatine should be classified as a β-glucosidase (β-D-glucoside glycohydrolase, EC 3.2.1.21). β-Glucosidases from fungi have already been reported[6]. However, this enzyme differs from them in having an unusually high degree of substrate specificity. Activity is apparently determined to a large extent by the enzyme's affinity for a portion of the substrate other than the β-1,2 linkage between the glucosyl moieties. If α-tomatine limits the ability of fungi to parasitize tomato, then the presence of a β-glucosidase with this specificity might confer a selective advantage upon that fungus.

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