Sterol Ester Hydrolase in *Fusarium oxysporum*¹

CLARENCE MADHOSINGH and WINSON ORR, Chemistry and Biology Research Institute¹, Research Branch, Agriculture Canada, Ottawa, K1A OCG, Canada

ABSTRACT

Two electrophoretically different forms of sterol ester hydrolase (EC 3.1.1.13) were obtained from the cytoplasmic extract of the mycelia of *Fusarium oxysporum*. The entities, estimated at 60,000 (I) and 15,000 (II) molecular weights, were obtained in Sephadex G100 column chromatography of the ammonium sulfate precipitate from the cytoplasmic extract. A third form III, 75,000 MW, was obtained from the culture filtrate. The activity of the enzyme was increased by Triton X-100 and was not inhibited by p-chloromercuribenzoate (PCMB), a sulfhydryl reagent. The enzymes I and II were inhibited differentially by NaCl. The optimal activities of forms I, II and III occurred at pH 4.8, pH 8.0 and pH 7.0, respectively. The apparent Km values of 7.7 × 10⁻⁵, 8.3 × 10⁻⁴ and 10.5 × 10⁻⁴, respectively, indicate a similar order of affinity for cholesteryl oleate at pH 7.1. The rate of hydrolysis of cholesteryl esters were in the order: linoleate > oleate > valerate > butyrate > acetate. Cholesteryl benzoate and palmitate were not hydrolyzed. The properties of the microbial enzyme are discussed in relation.

INTRODUCTION

Sterol ester hydrolase (esterase), EC 3.1.1.13, which catalyzes the hydrolysis and synthesis of fatty acid esters of sterols, has been examined extensively in a number of mammalian tissues primarily because of the involvement of sterol esters in atherosclerosis (1). There have been relatively few studies of the microbial enzyme (2-7) in spite of the widespread distribution and variety of sterols and fatty acids which occur particularly in the fungi. Steroids in fungi are the precursors of a number of hormones which influence growth, sexuality and the regulation of nucleic acid activity (8-10). Sterols have also been implicated in the survival and temperature tolerance of these organisms (11).

Lower growth temperatures appear to facilitate the production of more highly unsaturated fatty acids in the fungi (12-14). Miller and de la Roche (13) have demonstrated that the sterol and sterol ester contents of membranes of *Fusarium oxysporum* f. sp. *lycopersici*, are affected by temperature. These data suggest, therefore, an active sterol-fatty acid ester metabolism in *F. oxysporum*.

This study examines in *F. oxysporum*, the properties of sterol ester hydrolase, one of the principal enzyme systems in the metabolism of sterol esters.

MATERIALS AND METHODS

Materials

Labeled cholesteryl oleate [oleate-¹⁴C] 0.25 mCi was purchased from New England Nuclear Co. (Boston, MA); unlabeled cholesteryl esters, cholesterol, lecithin and sodium taurocholate were obtained from Supelco, Inc. (Belleville, PA). All other reagents were analytical grade. A microbial cholesterol esterase preparation was obtained from Boehringer Mannheim (Germany). Because of the patent, the source of the enzyme could not be obtained from the manufacturer.

Assay of Sterol Ester Hydrolase

The procedure essentially as described by Taketani et al. (2) was used for assaying the enzyme. Triton X-100 was used at 0.10% rather than at 0.3% in the assay as described by Taketani. Radioactive cholesteryl oleate was used as substrate at pH 7.1 unless mentioned otherwise. The chromatographic procedure, however, was modified as follows: After the chloroform extract of the reaction mixture was dried under N₂, aliquots in chloroform were applied quantitatively onto 20 × 20 cm Silica Gel 60 H thin layer plates (E.M. Reagent, E. Merck, Darmstadt, Germany). Standard cholesteryl oleate (25 µg) also was applied to the plates. The plates were developed by ascending chromatography in n-hexane/ethyl ether/acetic acid (70:30:1 by vol). The standards, sprayed with an anisaldehyde reagent consisting of acetic acid/sulfuric acid/p-anisaldehyde (50:1:0.5 by vol) located cholesteryl oleate, cholesterol and oleic acid at Rf 0.5, 0.15 and 0.33, respectively. The radioactivity of the samples was measured on a Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA).

One unit of cholesteryl ester hydrolase activity is defined as the amount of enzyme producing 1 µmol of free cholesterol or free oleic acid/min at 37 C. All hydrolase activity expressed in this study must be regarded as the net activity for the reasons described by Brock-
erhoff and Jensen (1). The same amount of enzyme, based on cholesteryl oleate activity, was used in all experiments where specifically indicated.

Assay for Substrate Specificity

Free cholesterol was measured colorimetrically by the method of Engelbrecht et al. (15). Ten μmol of the various ester substrates was incubated and the released cholesterol was extracted, separated and measured.

Extraction and Partial Purification of Cholesteryl Ester Hydrolase

The fungus *F. oxysporum* (DAOM 143566) is maintained at the Mycological Herbarium, Agriculture Canada, Ottawa, K1A OC6) was grown in 1-ℓ quantities of a modified Fries medium (16) at 30 C in shake cultures. After 40 hr incubation, the mycelia were filtered and washed. All extraction and purification procedures were performed at 0-4 C. For the cytoplasmic fraction, the filter-dried mycelia (134 g) were homogenized in 200 ml Tris buffer pH 7.4, 0.1 M with glass beads (268 g, 16-220 size, VirTis Co. Inc., Gardiner, NY) at high speed in the Omni Mixer (Servall Inc., Norwalk, CT) for 10 min. The fractions were obtained by differential centrifugation and by ammonium sulfate precipitation as illustrated in the following flow charts.

**Cytoplasmic enzyme preparation:**

- mycelium homogenate (48 g protein)

1. **centrifuge 30 X g (15 min)**
2. supernatant:
   - cell wall, etc.
   - **centrifuge 17,000 X g (20 min)**
   - supernatant:
     - mitochondria fraction
     - **centrifuge 100,000 X g (70 min)**
     - supernatant:
       - ppt
       - (NH₄)₂SO₄ precipitation
       - supernatant:
         - ppt
         - cytoplasmic fraction
       - Sephadex G100 chromatography

**Extracellular enzyme preparation:**

- 4-d, 96-hr culture (15 g protein)

1. **filter**
2. **centrifuge 17,000 X g (20 min)**
3. concentrate:
   - Amicon PM-10
4. 170 ml
5. (NH₄)₂SO₄ precipitation
6. supernatant:
7. (NH₄)₂SO₄ ppt
8. supernatant:
9. ppt
10. extracellular fraction
11. Sephadex G100 chromatography

Crystalline, enzyme-grade (NH₄)₂SO₄ was added slowly to the gently stirred cytoplasmic and extracellular fractions to achieve 100% saturation. Only precipitates obtained between 75 and 100% saturation in the mycelium preparation and 35% saturation in the extracellular preparation demonstrated sterol ester hydrolase activity. The resulting precipitates were chromatographed on a Sephadex G100 column. Components in the chromatographic fractions were separated by polyacrylamide gel disc electrophoresis at 4 C using Tris-glycine buffer at pH 8.3 and a 10.5% acrylamide gels in 75 x 5 mm glass tubes at 3 mA/gel with bromophenol blue as the tracking dye. Electrophoretic samples contained 1-5% protein, which was determined by the method of Lowry et al. (17).

Cholesteryl ester hydrolase activity on the gels was detected by the Szécsi et al. method (18).

Molecular Weight Estimation

The molecular weight of the enzyme was estimated by column chromatography on a Sephadex G100 column as described by Ackers (19) and by sucrose density gradient centrifugation according to Martin and Ames (20) and Hyun et al. (21).

The molecular weight markers used for comparison with the enzyme were myoglobin (16,890 MW), ovalbumin (45,000 MW), bovine serum albumin (B.S.A. 68,000 MW), gamma