Some characteristics of peroxidase secreted by cotton ovule cultures

Jay E. Mellon

US Department of Agriculture, Agriculture Research Service, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179, USA

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ABSTRACT

Cotton (Gossypium hirsutum L.) ovule cultures secreted a soluble peroxidase into the surrounding medium, resulting in a 200-fold increase in this activity during the 30-day growth period. The peroxidase activity was thermostable from 4°C to 60°C and displayed a pH optimum of 5.5 to 6.0. The ovule peroxidase was susceptible to periodate treatment and very resistant to protease digestion. The data suggest that the peroxidase activity is a glycoprotein. Interpretation of peroxidase data may be complicated by the presence of phenol oxidase activity in the same preparations. The presence of phenol oxidases was ruled out by the inaction of a tyrosinase-specific inhibitor, tropolone.

ABBREVIATIONS

PMSF, phenylmethylsulfonyl fluoride

INTRODUCTION

Peroxidase secretion into the growth medium has been described in cell suspension cultures, including those of carrot (Chibbar et al. 1984), peanut (van Huystee and Turcon 1973), potato (Bredemeijer et al. 1985) and spinach (Fry 1980). Peroxidase secretion by in vitro fertilized cotton ovule cultures has been observed in this laboratory during the course of an investigation of ovule-specific proteins (Mellon 1985). Since soluble isoperoxidases are elicited by fungal infection in cotton bolls in vivo (Mellon and Lee 1985), the cotton ovule system appears to offer a convenient method of investigating a specific stress-induced protein. This report summarizes some properties of the peroxidase activity secreted by cotton ovule cultures.

MATERIALS AND METHODS

Cotton (G. hirsutum L. 'Stoneville 208 7A' standard and glandless) plants were maintained in standard greenhouse conditions. In vitro fertilized cotton ovule cultures were initiated and maintained according to the method of Stewart and Hsu (1977).

Culture medium derived from 60-day-old cotton ovule cultures provided the crude preparations of peroxidase activity. The medium was filtered in vacuo before storage at 4°C.

Protein concentrations in ovule medium preparations dialyzed against 0.02 M Tris-HCl buffer, pH 8.0 were estimated with the bicinchoninic acid technique (Smith et al. 1985; Pierce Chemical Company), using bovine serum albumin as a standard. This new protein quantitation method provided reliable results without the interference from buffer components or non-proteaceous impurities observed with other protein assays.

Peroxidase was assayed by the method of Heidrich et al. (1983). The standard assay mixture contained 0.94 mM H₂O₂ and 6.25 mM guaiacol in 62.5 mM McIlvaine's citrate-phosphate buffer, pH 5.5, in a final volume of 3.2 ml. One unit of peroxidase activity will oxidize 1 μM guaiacol per minute to tetra-guaiacol.

The peroxidase preparation for the pH optimum and inhibitor studies was obtained by dialyzing (MW cutoff 6-8 kd) ovule culture medium (60 days post culture initiation) against 0.02 M Tris-HCl, pH 8.0. A series of citrate-phosphate buffers of equal ionic strength with a pH range of 2.5 to 7.6 was utilized in the pH optimum study and substituted for the standard buffer (citrate-phosphate, pH 5.5). For the rubeanic acid inhibition study, the standard assay mixture contained 5% (v/v) 2-propanol to maintain inhibitor solubility. A 100 mM stock solution of rubeanic acid was prepared in 2-propanol-acetone (1:1).

Peroxidase preparations for pronase and periodate digestion studies were obtained by dialysis of the ovule culture medium against 0.05 M phosphate buffer, pH 7.0. Pronase E (0.1-1.0 mg/ml; Sigma Chemical Co.) digestions were conducted at 37°C for 24 h before termination by the addition of PMSF to 1 mM. Pronase incubation mixtures contained 5% (v/v) 2-propanol before the addition of PMSF to maintain inhibitor solubility. The terminated protease reactions were re-dialyzed against the given phosphate buffer prior to peroxidase analysis. Periodate reaction mixtures containing 0.1 M NaIO₄ were incubated at 25°C for 24 h. A control incubation mixture contained 2 M glycerol prior to the addition of NaIO₄. All reaction samples were re-dialyzed against the phosphate buffer (pH 7.0) before peroxidase analysis.

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RESULTS

Peroxidase secretion in ovule cultures was examined as a potential parameter to reflect genetic differences between glanded and glandless cotton. Similar secretion kinetics were measured for ovule cultures derived from either glanded or glandless cotton (Fig. 1). Secretion started slowly, but increased rapidly after the cultures were 10 days old. After initial detection at 5 days, activity levels increased to yield a 200-fold increase at 30 days. The greatest peroxidase secretion rate corresponded to the developmental period when secondary thickening occurs in cotton fibers (after 20 days). Sixty-day cultures commonly exhibited activity levels of 200 to 300 units per ml and protein concentrations of 75 to 95 µg per ml (± 8 µg/ml).

The secreted peroxidase activity displayed considerable thermostability (Fig. 2), remaining fully active until temperatures of greater than 60°C were attained. Ovule peroxidase demonstrated a pH optimum of 5.5 to 6.0 (Fig. 3) and rapidly lost activity below pH 4.0.

Treatment of the ovule medium with periodate virtually eliminated peroxidase activity (Table 1). Inclusion of glycerol in the reaction mixture prior to the addition of periodate did not protect the peroxidase activity. The pH of the control reaction after the addition of periodate was 6. A white precipitate was observed in the periodate reaction mixtures at the end of the incubation period and is believed to represent denatured protein.

Peroxidase activity proved to be remarkably resistant to digestion by a nonspecific protease, Pronase E (Table 2). This protease preparation was very active against bovine serum albumin in standard assay conditions (data not shown). The protease inhibitor (PMSF) used to stop the reactions caused a slight inhibition of the peroxidase activity (Table 2).

Inhibitor studies were conducted to investigate the possibility of the presence of phenol oxidase(s) in the ovule medium preparations, since both peroxidase and phenol oxidase activities may occur in crude preparations. Because copper chelators are effective inhibitors of phenol oxidase, the chelator rubeanic acid was utilized to identify the presence of phenol.