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SECRETION OF ASCORBATE OXIDASE BY SUSPENSION-CULTURED PUMPKIN CELLS

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Key Word Index—*Cucurbita* spp.; Cucurbitaceae; pumpkin; secretion; glycoprotein; ascorbate oxidase.

Abstract—Ascorbate oxidase is released into the medium in pumpkin (*Cucurbita* sp.) cell suspension cultures. Alcohol dehydrogenase and glucose-6-phosphate dehydrogenase, cytosolic enzymes, and catalase, microbody enzyme, were not detected in the medium, whereas peroxidase, which is known to be a secretory protein, was detectable. Immunological blotting showed that the M_r of ascorbate oxidase secreted into the medium was identical to that of the enzyme purified from pumpkin fruit tissue. Purified ascorbate oxidase on acrylamide gel was stained by periodic acid–Schiff method. Thus, ascorbate oxidase is probably a secretory glycoprotein.

INTRODUCTION

Recently, much attention has been focused on the production by cultured plant cells of useful metabolites such as alkaloids, steroids, vitamins and pigments, and enzymes such as horseradish peroxidase. In this case, it is convenient that the useful metabolites and enzymes are secreted by cultured plant cells. In regard to enzymes, peroxidase [1, 2], phosphatase [3, 4], glucosidase [5], laccase [6] and others have been suggested to be secreted by cultured plant cells.

Ascorbate oxidase (E. C. 1.10.3.3) is a copper-containing enzyme widespread in plant tissues and involved in secondary metabolism [7–9]. The enzyme has been recently used as a valuable reagent for clinical and food analyses of L-ascorbic acid [10, 11], although its precise biological function has not been clarified. We have studied the ascorbate oxidase in cultured pumpkin cells. In a previous paper [12], we reported that ascorbate oxidase activity rapidly increased during growth of cultured pumpkin cells. Furthermore, we showed that ascorbate oxidase in the cultured cells was induced by copper, a prosthetic metal of the enzyme [13]. In the present study, we show that ascorbate oxidase is secreted by cultured pumpkin cells and furthermore suggest that the enzyme is a glycoprotein.

RESULTS AND DISCUSSION

Since ascorbate oxidase is a valuable enzyme which is used for the determination of L-ascorbic acid, a higher productivity of the enzyme is desirable. In a previous paper [12], ascorbate oxidase was shown to be produced from cultured pumpkin cells. Furthermore, ascorbate oxidase was suggested to be induced by copper, a prosthetic metal of the enzyme [13]. For more efficient production of the enzyme, the secretion of ascorbate oxidase by cultured pumpkin cells is also expected. In the cell suspension cultures grown in media containing 0, 0.01, 0.1, 1 or 10 μM copper sulphate ($CuSO_4$) for five or ten days, ascorbate oxidase activity in the medium was compared with that in the cells (Fig. 1). Ascorbate oxidase activity in the cells clearly depended on the copper sulphate concentration in the culture medium. In the same manner, ascorbate oxidase activity in the medium was also increased by adding copper to the fresh culture medium. In the cultures grown in the medium containing 0, 0.01 and 0.1 μM $CuSO_4$, ascorbate oxidase activity in the medium was more than twice that in the cells. In the case of 1 μM $CuSO_4$, the activity in the medium was almost the same that in the cells. Ascorbate oxidase activity in the medium, in the presence of 10 μM $CuSO_4$, was a little lower than that in the cells. The decrease in the rate of activity in the medium to that in the cells with increase in $CuSO_4$ concentration may be due to a limit on enzyme secretion. At any rate, a large amount of ascorbate oxidase was found to be released into the culture medium. On the other hand, the activities of alcohol dehydrogenase and glucose-6-phosphate dehydrogenase, cytosolic enzymes which are easily released from damaged cells, was not detected in the culture medium. Furthermore, the activity of catalase, a microbody enzyme which is also detected in the cytoplasmic fraction [14], was also undetected in the medium. The activity of peroxidase, which is suggested to be a secretory enzyme, was detected in very small quantities in the medium. Thus, the possibility that ascorbate oxidase activity in the medium is from damaged cells can be discounted. Finally, ascorbate oxidase is suggested to be secreted by cultured pumpkin cells. The subcellular localization of ascorbate oxidase has not been established yet, although there have been some reports that the enzyme is localized in the cytoplasm [15], microsomes, vacuole [16] or cell wall [17]. The present study indicated that, in pumpkin cells, ascorbate oxidase was an extracellular enzyme. At present, we have only attempted to purify the extracellular ascorbate oxidase from the culture medium. Additionally, in the cells, the activities of four enzymes other than ascorbate oxidase were independent of copper sulphate concentration in the culture medium, although their activities per g fresh weight in the cells increased after the transfer of cells to fresh medium (Fig. 1). This suggest that the induction of ascorbate oxidase by copper may not be caused by a secondary effect.

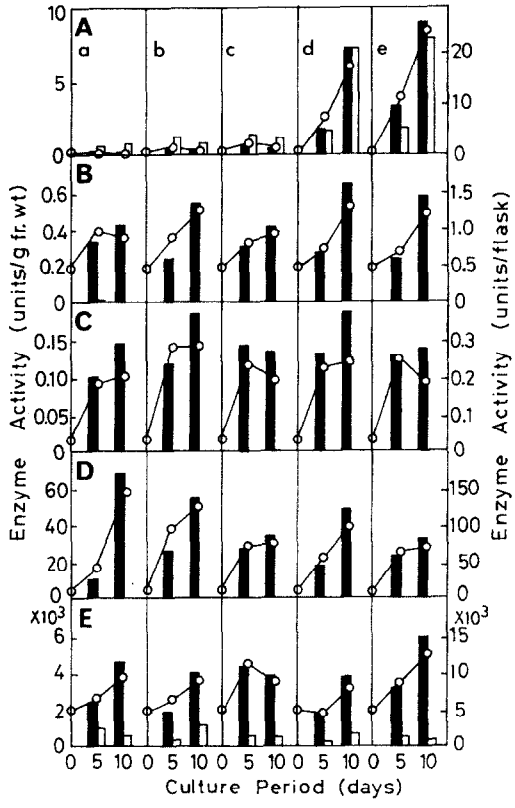

Fig. 1. Ascorbate oxidase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, catalase and peroxidase activities in cells and in the medium after pumpkin cell suspension cultures in the presence of $CuSO_4$ of various concentrations. Pumpkin cells were inoculated in Murashige and Skoog's liquid medium containing 0 (a), 0.01 (b), 0.1(c), 1 (d) or 10 μM $CuSO_4$ (e) and cultured at 25°. The cultures were harvested at 5 or 10 days, and the cells were separated from the cultures and homogenized as described in experimental. The cell homogenate and the culture medium were assayed for ascorbate oxidase (A), alcohol dehydrogenase (B), glucose-6-phosphate dehydrogenase (C), catalase (D) and peroxidase activities (E). o, enzyme activity expressed as units per g fr. wt in the cells; black bar, enzyme activity expressed as units per flask in the cells; white bar, enzyme activity expressed as units per flask in the medium.

In a previous paper [13], we showed that the M_r of ascorbate oxidase in pumpkin callus was the same as that in pumpkin fruit tissue, by the method of immunological blotting with anti-ascorbate oxidase antibody. When the culture medium was subjected to SDS-polyacrylamide gel electrophoresis, the main band was detected in the position corresponding to the polypeptide band of ascorbate oxidase purified from pumpkin fruit tissue. Furthermore, immunological blotting with anti-ascorbate oxidase antibody showed that it was the immunoreactive polypeptide band, suggesting that it is an ascorbate oxidase polypeptide band. Thus, the M_r (64 000) of the subunit of ascorbate oxidase secreted into the culture medium was identical to that of the same enzyme in pumpkin fruit tissue.

Most secretory proteins have been suggested to be glycoproteins [18]. For instance, Bligny and Douce [6] have reported that a laccase-type polyphenol oxidase secreted by sycamore cells contains 45% carbohydrate. We investigated whether ascorbate oxidase was a glycoprotein, or not. Our present preparation of ascorbate oxidase contains no contaminating protein, considering that a single polypeptide band was detected after SDS-polyacrylamide gel electrophoresis. The purified ascorbate oxidase preparation on the gel was stained by periodic acid–Schiff method, suggesting that the enzyme is a glycoprotein. Furthermore, ascorbate oxidase secreted into the culture medium was suggested to be a glycoprotein, since the polypeptide band corresponding to ascorbate oxidase in the culture medium was also stained by periodic acid–Schiff. This is the first report of ascorbate oxidase being a glycoprotein. Takahashi *et al.* [19] have described a structural study of oligosaccharides of laccase from sycamore cells. A structural study of oligosaccharides of ascorbate oxidase will be also required. Furthermore, the present study raise the problem of the passage of ascrobate oxidase protein across the cytoplasmic membrane. Akazawa and Hara-Nishimura [20] have discussed a possible role of the Golgi system in the biosynthesis and secretion of proteins in plant cells. Further studies will be required to investigate the properties of extracellular ascorbate oxidase and to elucidate the process of extracellular secretion of ascorbate oxidase by cultured pumpkin cells.

EXPERIMENTAL

Plant material and culture methods. Pumpkin (*Cucurbita* sp, Ebisu Nankin) fruits were purchased from a local market and stored at *ca* 15° until use. Callus was induced from the sarcocarp tissue as described in ref. [12]. Suspension-cultures were established by inoculating callus cells, which had been subcultured at *ca* 4-week intervals for more than one year, into a 200-ml Erlenmeyer flask containing 50 ml of the Murashige and Skoog's liquid medium with 1.0 mg/l 2,4-D, 0.1 mg/l kinetin and 3% (w/v) sucrose. These cultures were agitated on a rotary shaker at 100 rpm at 25°, and maintained by transferring *ca* 1 g (fr. wt) of the cells to fresh liquid medium at *ca* 4-week intervals.

Preparation of cell homogenate. Cultured cells sepd from suspension cultures by filtering the medium through nylon mesh were homogenized in 0.05 M Tris–HCl buffer, pH 7, with a glass homogenizer. The homogenates thus prepd were squeezed through nylon gauze and filtered through a Toyo No.2 filter. The filtrates and culture medium were assayed for enzyme activities. All procedures were performed at *ca* 4°.

Purification of ascorbate oxidase. Ascorbate oxidase prepn from pumpkin was purchased from Boehringer Mannheim Co. The enzyme soln (*ca* 1 mg protein) was fractionated with satd $(NH_4)_2SO_4$ soln at pH 7, and the proteins pptd between 40 and 80% satn were collected and dissolved in 0.5 ml of 0.01 M Tris–HCl buffer, pH 7. The enzyme soln thus obtained was applied to a Sepharose 6B column (1.5 × 50 cm) that had been equilibrated with the above buffer; proteins were eluted from the column with the same buffer (flow rate: *ca* 5 ml/hr). Fractions (0.6 ml each) were collected, and the most active fraction was used as the purified prepn. All procedures were performed at *ca* 4°.

Polyacryamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was run in a gel containing 10% (w/v) polyacrylamide and 0.1% (w/v) SDS by the method of ref. [21]. The sample was heated at 100° for 10 min in 0.01 M Tris–HCl buffer, pH 7, containing 2% (w/v) SDS, 2% (w/v) mercaptoethanol and 5% (w/v) glycerol before electrophoresis. The gels were stained with Coomassie Brilliant Blue R. Myosin (M_r 205 000), β-

galactosidase (M_r 116000), phosphorylase (M_r 97400), albumin bovine (M_r 66000), albumin egg (M_r 45000) and carbonic anhydrase (M_r 29000) were used as markers.

Immunological blotting. Antiserum against the purified ascorbate oxidase prepn was prepd as described in ref. [13]. The immunological blotting technique was performed as described in ref. [22] with slight modifications. Each sample first underwent electrophoresis on slab polyacrylamide (10%, w/v) gel containing 0.1% (w/v) SDS as described above. After electrophoresis, the gel was covered with a sheet of nitrocellulose paper which had been wetted with 0.02 M Tris containing 0.19 M glycine, 0.1% SDS and 20% MeOH (pH 8.3). The polypeptides in the gel were transferred to nitrocellulose paper by electrophoresis with a constant current of 100 mA for 2 hr using a electrophoretic transblot apparatus. The nitrocellulose paper was shaken first in saline soln (0.02 M Tris–HCl, pH 7.8, and 0.15 M NaCl) containing 2% BSA, then in the same saline soln containing the immunoglobulin G fraction at 25° for 1 hr, then at 4° overnight. Following washing, the paper was shaken in saline soln containing horseradish peroxidase linked protein A (Bio-Rad Lab.) at 25° for 1 hr and afterwards washed again with saline soln and dist H_2O. Horseradish peroxidase colour development soln (Bio-Rad Lab.) was added and incubated with the paper at 25°. When the colour band was detected, the paper was washed with dist. H_2O.

Glycoprotein staining on acrylamide gel. SDS-polyacrylamide gel electrophoresis was run in a gel containing 10% (w/v) polyacrylamide and 0.1% (w/v) SDS as described above. The carbohydrate moiety of the ascorbate oxidase on the gel was detected by periodic acid–Schiff method as described in ref. [23].

Assays of enzyme activities. Ascorbate oxidase activity was assayed by the method of ref. [24], alcohol dehydrogenase and glucose-6-phosphate dehydrogenase activities by the methods of refs [25,26], respectively, catalase activity by the method of ref. [27] and peroxidase activity by the method of ref. [28].

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