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Marked Increase in Ascorbate Oxidase Protein in Pumpkin Callus by Adding Copper[1]

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ABSTRACT

Ascorbate oxidase from pumpkin (*Cucurbita* sp.) was purified from a commercially available preparation. A single polypeptide band with M_r 64,000 was detected after sodium dodecylsulfate-polyacrylamide gel electrophoresis of the purified enzyme. In double immunodiffusion tests, antiserum against the purified preparation formed a single precipitin line with the crude extract from pumpkin fruit tissue or the callus as well as with the purified preparation. Immunological blotting method showed that mol wt of ascorbate oxidase subunit in pumpkin callus was the same as that of the purified preparation. Analysis with the single radial immunodiffusion method showed that the increase in ascorbate oxidase activity during the growth of pumpkin callus correlated with an increase in the enzyme protein. Furthermore, enzyme protein in the callus grown in the presence of 10 micromolar $CuSO_4$ for 2 weeks was about eight times that grown in the presence of 0.1 micromolar $CuSO_4$. The synthesis of ascorbate oxidase in pumpkin callus may be induced by copper, a prosthetic metal of the enzyme, or copper may help stabilize the enzyme against proteolytic breakdown.

Ascorbate oxidase (EC 1.10.3.3) is a copper-containing enzyme that occurs in many plant tissues and plays a role in secondary metabolism (9, 18). The precise biological function of ascorbate oxidase has not been clarified, although it has been reported that the enzyme may participate in redox system involving ascorbic acid (19).

We studied the ascorbate oxidase in cultured pumpkin cells, and in a previous paper (4), reported that ascorbate oxidase activity rapidly increased during growth of pumpkin callus. We showed that ascorbate oxidase activity in pumpkin callus could be markedly increased by adding copper, a prosthetic metal of the enzyme. There have been only a few reports that the enzyme in cultured plant cells is induced by its prosthetic metal. Palacco (15) has reported that urease activity in soybean tissue cultures is increased by adding nickel, which is a component of urease. Ikeda *et al.* (5) have also reported that Cyt aa_3 content in cultured tobacco cells increases twofold after subculturing seven times in high copper concentration medium. Recently, Delhaize *et al.* (3) have reported that the amount of diamine oxidase protein in clover leaves is controlled by the copper concentrations of the leaves. Jones and Carbonell (6) have reported that the amount of α-amylase in aleulone layers of barley is controlled by calcium, the metal cofactor of the enzyme. Furthermore, in *Chlamydomonas reinhardtii*, the amount of plastocyanin apoprotein has been shown to be regulated by the copper concentration of the medium (20). We now report that the increase in ascorbate oxidase activity during the growth of pumpkin callus correlates with an increase in the enzyme protein, and that the marked increase in ascorbate oxidase activity by adding copper also correlates with an increase in the enzyme protein, suggesting that copper controls the amount of ascorbate oxidase protein.

MATERIALS AND METHODS

Plant Material and Culture Methods. Pumpkin (*Cucurbita* sp., Ebisu Nankin) fruits were purchased from a local market and stored at about 15°C until use. Callus was induced from the sarcocarp tissue as described in a previous paper (4). Discs (8 mm in diameter, 2 mm thick) prepared using a cork borer and a surgical blade under aseptic condition were cultured in a 100 ml Erlenmeyer flask (4 discs/flask) containing 50 mL of Murashige and Skoog's basal medium with 1.0 mg/L 2,4-D, 0.1 mg/L kinetin, 3% (w/v) sucrose, and 0.8% (w/v) agar. The material was grown at 25°C in the dark, and the cultures were maintained by transferring about 1 g of the callus every 4 weeks.

Preparation of Crude Extract. Pumpkin callus was homogenized in 0.05 M Tris-HCl buffer, pH 7.0, with a glass homogenizer. Pumpkin fruit tissues were cut into pieces and homogenized in the same buffer with a Waring blender and glass homogenizer. The homogenates thus prepared were squeezed through nylon gauze and filtered through a Toyo No. 2 filter. The filtrates were used as crude extracts. All the procedures were performed at about 4°C.

Purification of Ascorbate Oxidase. Ascorbate oxidase preparation from pumpkin was purchased from Boehringer Mannheim Co. The enzyme solution (about 1 mg protein) was fractionated with saturated $(NH_4)_2SO_4$ solution at pH 7.0, and the proteins precipitated between 40 and 80% saturation were collected and dissolved in 0.5 ml of 0.01 M Tris-HCl buffer, pH 7.0. The enzyme solution 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: about 5 ml/h). Fractions (0.6 ml each) were collected, and the most active fraction was used as the purified preparation. The above procedures were performed at about 4°C.

PAGE. SDS-PAGE was run in a gel containing 10% (w/v) polyacrylamide and 0.1% (w/v) SDS by the method of Laemli (8). The sample was heated at 100°C for 10 min in 0.01 M Tris-HCl buffer, pH 7.0, containing 2% (w/v) SDS, 2% (w/v) mercaptoethanol, and 5% (w/v) glycerol before the electrophoresis. The gels were stained with Coomassie brilliant blue R. Myosin (M_r 205,000), β- galactosidase (M_r 116,000), phosphorylase (M_r 97,400), albumin bovine (M_r 66,000), albumin egg (M_r 45,000), and carbonic anhydrase (M_r 29,000) were used as mol wt standards.

Immunological Methods. Antiserum against the purified as-

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corbate oxidase preparation was produced in a rabbit by injecting twice at an interval of about 20 days with a mixture of the purified preparation (about 100 μg protein) and complete Freund's adjuvant, followed by an injection of the mixture (about 20 μg protein) after 1 week. An immunoglobulin G fraction was prepared from the antiserum by fractionation with $(NH_4)_2SO_4$. Double immunodiffusion tests were performed by the method of Ouchterlony and Nilsson (14). The plates were prepared with 0.9% (w/v) agarose containing 0.01 M Tris-HCl (pH 7.8), 0.15 M NaCl and 0.1% (w/v) NaN_3. After 20 μl each of samples were added to wells (4.2 mm in diameter and 2 mm in depth), the plates were incubated at 25°C for 2 days. Immunoprecipitation was carried out by incubation of either the purified ascorbate oxidase preparation or the crude extract from pumpkin callus with arbitrary volumes of immunoglobulin G fraction at 25°C for 2 h. The incubation mixtures were centrifuged at 9000*g* for 20 min, and the supernatants were assayed for ascorbate oxidase activity. The immunological blotting technique was performed as described by Towbin *et al.* (17), with slight modifications. Each sample first underwent electrophoresis on the 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% methanol (pH 8.3). The polypeptides in the gel were transferred to nitrocellulose paper by electrophoresis with a constant current of 100 mamp for 1 h using an electrophoretic transblot apparatus. The nitrocellulose paper was shaken first in a saline solution (0.02 M Tris-HCl, at pH 7.8, and 0.15 M NaCl) containing 2% bovine serum albumin, then in the same saline solution containing the immunoglobulin G fraction at 25°C for 1 h, then at 4°C overnight. Following washing, the paper was shaken in the saline solution containing horseradish peroxidase linked protein A (Amersham Co.) at 25°C for 1 h and afterward washed again with the saline solution and distilled water. Horseradish peroxidase color development solution (Amersham Co.) was added and incubated with the paper at 25°C. When the color band can be detected, the paper was washed with distilled water. Single radial immunodiffusion method described by Mancini *et al.* (10) was used to determine the amount of ascorbate oxidase protein. The plate contained 0.9% (w/v) agar, 0.01 M Tris-HCl (pH 7.8), 0.15 M NaCl, 0.01% (w/v) NaN_3, and 0.05% (v/v) antiserum against ascorbate oxidase. Wells (4.2 mm in diameter and 2 mm in depth) contained 20 μL each of the samples tested. After incubation for 3 days at 25°C, the plate was washed with 0.01 M Tris-HCl (pH 7.8) containing 0.15 M NaCl, 0.01% (w/v) NaN_3 and 0.4% (v/v) Triton X-100, and stained with Coomassie brilliant blue R.

Assay of Ascorbate Oxidase Activity. Ascorbate oxidase activity was assayed at 25°C by following the decrease in A_{265} of the reaction mixture containing 0.05 M K-phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.002% metaphosphoric acid, 0.15 mM L-ascorbic acid, and enzyme solution in a final volume of 3.0 mL, according to the method of Oberbacher and Vines (13). One unit

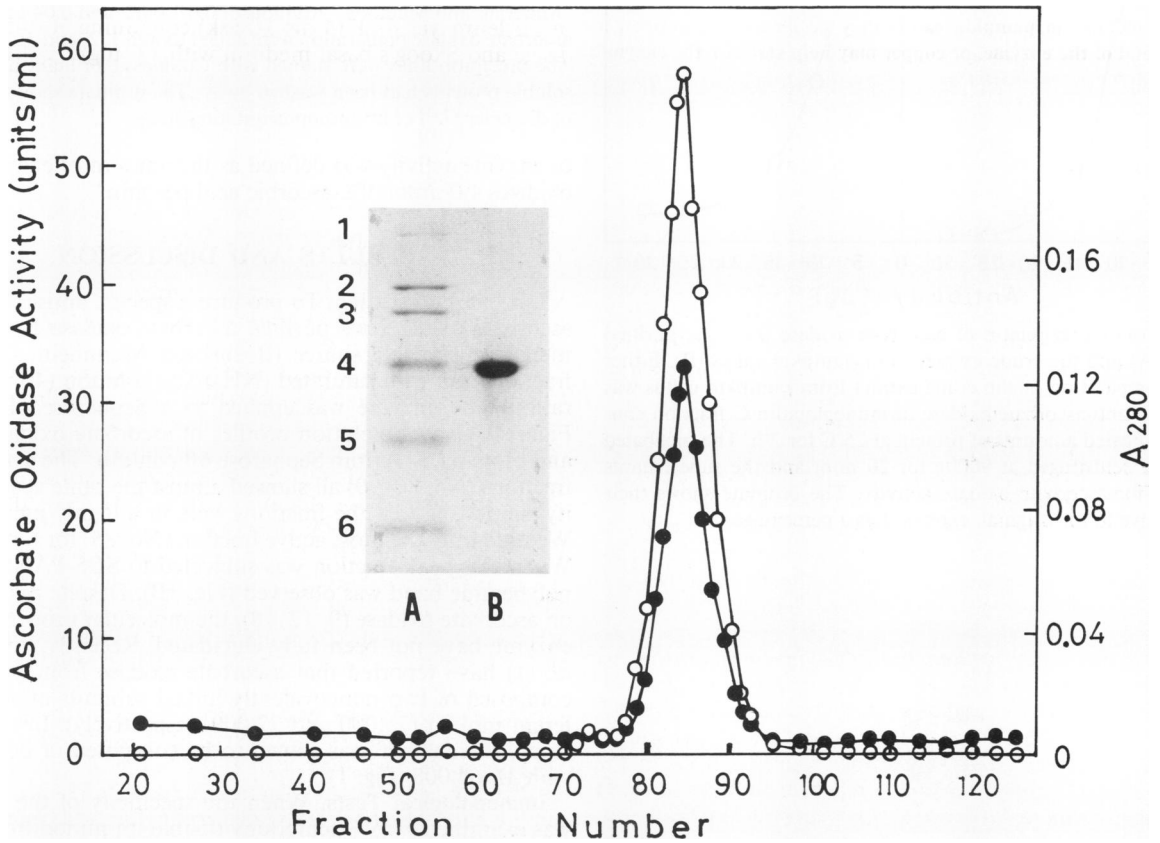

FIG. 1. Sepharose 6B column chromatography of ascorbate oxidase preparation, and SDS-PAGE of the peak fraction. The partially purified preparation after a $(NH_4)_2SO_4$ fractionation was applied on a Sepharose 6B column as described in "Materials and Methods," and fractions (0.6 ml each) were assayed for ascorbate oxidase activity (○) and protein (A_{280}, ●). The peak fraction and mol wt standards were subjected to a 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS as described in "Materials and Methods." Lane A, mol wt standards; (1) myosin (205,000), (2) β-galactosidase (116,000), (3) phosphorylase (97,400), (4) albumin bovine (66,000), (5) albumin egg (45,000), and (6) carbonic anhydrase (29,000). Lane B; purified ascorbate oxidase preparation.

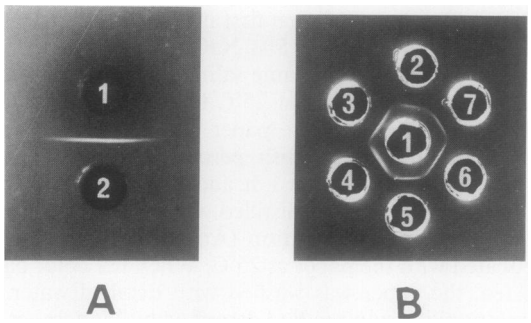

FIG. 2. Double immunodiffusion tests. Double immunodiffusion tests were performed as described in "Materials and Methods." A, Well 1, anti-ascorbate oxidase immunoglobulin G fraction (about 10 μg protein); well 2, purified ascorbate oxidase preparation (about 1 μg protein), B, Center well (well 1), anti-ascorbate oxidase immunoglobulin G fraction (about 5 μg protein); wells 2 and 5, purified ascorbate oxidase preparation (about 0.5 μg protein each); wells 3 and 6, crude extract from pumpkin fruit tissue; wells 4 and 7, crude extract from pumpkin callus.

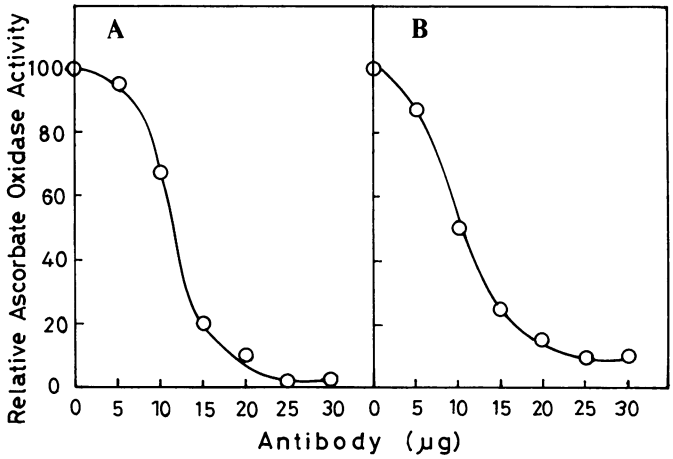

FIG. 3. Immunoprecipitatin of ascorbate oxidase from the purified preparation (A) and the crude extract from pumpkin callus (B). Either the purified preparation or the crude extract from pumpkin callus was incubated with anti-ascorbate oxidase immunoglobulin G fraction containing the indicated amounts of protein at 25°C for 2 h. The incubated mixtures were centrifuged at 9000*g* for 20 min, and the supernatants were assayed for ascorbate oxidase activity. The ordinate shows their activities relative to the original, expressed as a percentage.

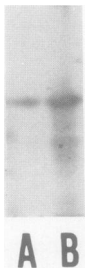

FIG. 4. Immunological blotting of SDS-polyacrylamide gels applied with purified ascorbate oxidase preparation (A) and crude extract from pumpkin callus grown in the presence of 10 μM $CuSo_4$ (B). Immunological blotting was performed as described in "Materials and Methods."

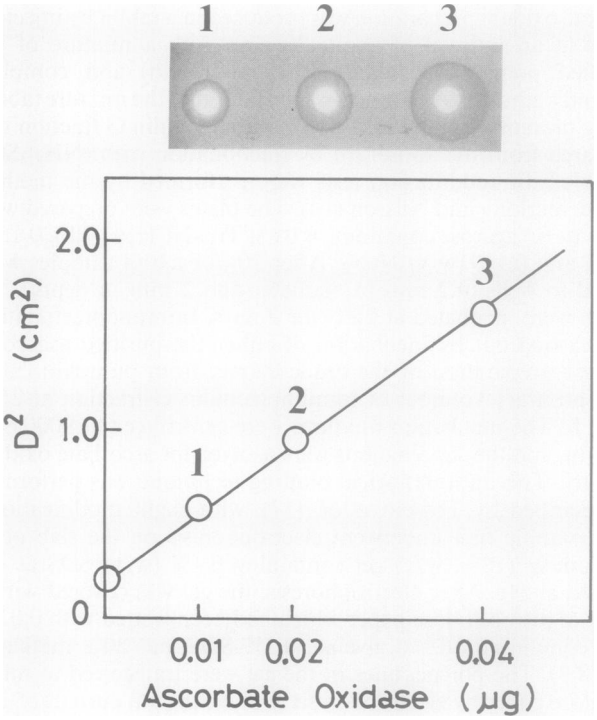

FIG. 5. Single radial immunodiffusion of purified ascorbate oxidase preparation. Single radial immunodiffusion was done as described in "Materials and Methods." Aliquots of 0.01, 0.02, and 0.04 μg of purified ascorbate oxidase preparation were added to well 1, 2, and 3, respectively. The precipitin lines were stained with Coomassie brilliant blue R, after soluble proteins had been washed away. The ordinate shows the square of diameter (D^2) of immunoprecipitating circle.

of enzyme activity was defined as the amount of enzyme which oxidizes 1.0 μmol of L-ascorbic acid per min.

RESULTS AND DISCUSSION

Enzyme Purification. To produce a specific antiserum against ascorbate oxidase, we purified ascorbate oxidase from a commercially available source (Boehringer Mannheim Co.). After fractionation with saturated $(NH_4)_2SO_4$ solution (40–80% saturation), the enzyme was applied to a Sepharose 6B column. Figure 1 gives the elution profiles of ascorbate oxidase activity and protein (A_{280}) from Sepharose 6B column. The activity-peak fractions (No. 80–90) all showed almost the same specific activity, suggesting that the fractions were in a highly purified state. We used only the most active fraction (No. 85) for further work. When this peak fraction was subjected to SDS-PAGE, a single polypeptide band was observed (Fig. 1B). Despite many reports on ascorbate oxidase (9, 12, 18), the molecular properties of this enzyme have not been fully elucidated. Recently, Avigliano *et al.* (1) have reported that ascorbate oxidase from pumpkin is composed of two noncovalently linked subunits of slightly different mol wt (75,000 and 72,000, respectively). In the present study, the enzyme was shown to be composed of polypeptides with M_r 64,000 (Fig. 1).

Immunological Tests. When the specificity of the antiserum was examined with Ouchterlony double immunodiffusion tests, the antiserum formed a single precipitin line with the antigen (Fig. 2A). Furthermore, the antiserum also formed a single precipitin line with the crude extract from pumpkin fruit tissue or pumpkin callus (Fig. 2B). The precipitin lines formed with the purified preparation and each crude extract were fused to one another (Fig. 2B), suggesting that they were identical in their

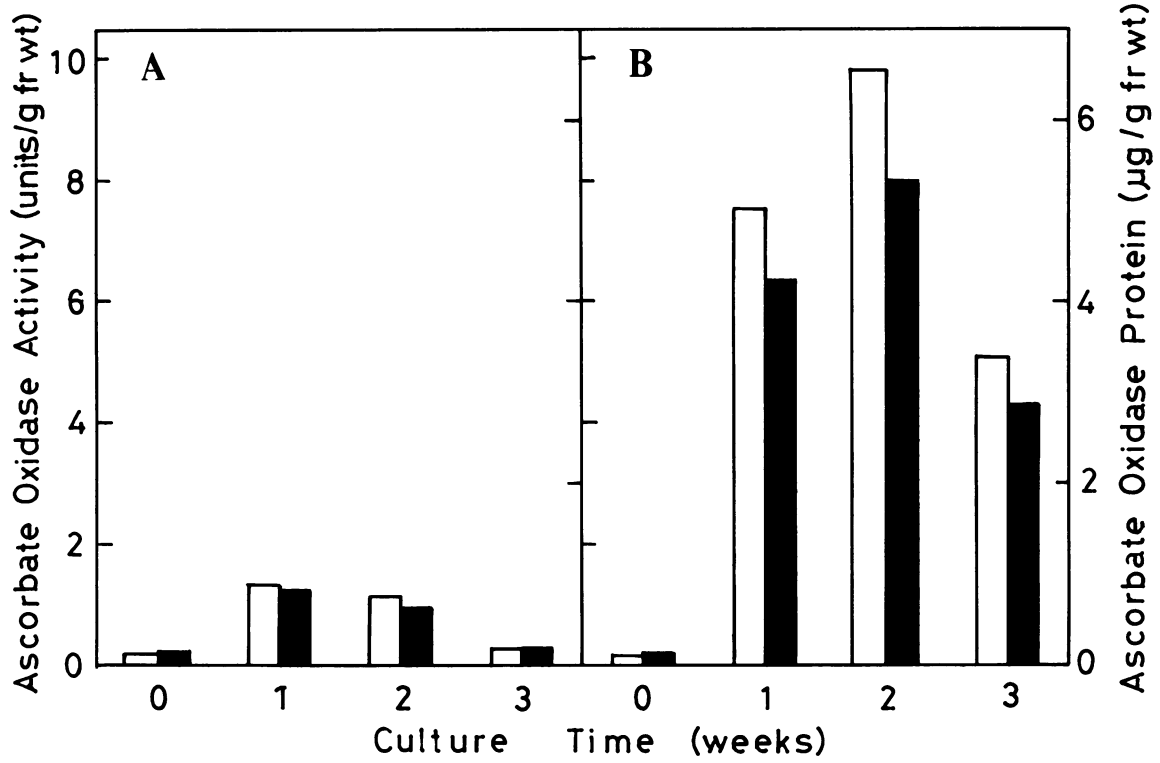

FIG. 6. Changes in the activity of ascorbate oxidase and the amount of immunoreactive protein during growth of pumpkin callus. Pumpkin callus was inoculated into each flask containing Murashige and Skoog's agar medium with 0.1 (A) or 10μM $CuSo_4$ (B), and cultured at 25°C. The callus was harvested at the indicated times and homogenized as described in "Materials and Methods." The crude extracts were assayed for the enzyme activity and the amount of immunoreactive protein. White bar, ascorbate oxidase activity expressed as units per g fresh weight; black bar, the amount of immunoreactive protein determined by single radial immunodiffusion method and expressed as μg per g fresh weight.

immunological properties. Purified ascorbate oxidase was almost completely precipitated by the antiserum (Fig. 3A), while about 90% of ascorbate oxidase in the crude extract from pumpkin callus was precipitated by the antiserum (Fig. 3B). The reason why about 10% of ascorbate oxidase in pumpkin callus could not be precipitated by antiserum is not yet known. When the purified ascorbate oxidase preparation and the crude extract from the pumpkin callus grown in the presence of 10 μM $CuSO_4$ were electrophoresed on a polyacrylamide gel containing 0.1% (w/v) SDS and the gel underwent immunological blotting (Fig. 4), the mobility of the major immunoreactive protein in pumpkin callus did not differ from that of the purified preparation although the minor immunoreactive proteins different from the purified preparation in the mobility were detected. This suggests that ascorbate oxidase in pumpkin callus may also be composed of polypeptides with M_r 64,000, as well as the purified preparation.

Change in Amount of Ascorbate Oxidase Activity and Protein in Callus. As reported previously (4), ascorbate oxidase activity in pumpkin callus rapidly increased after transfer to the fresh medium, and reached a maximum in the early logarithmic phase of growth. Furthermore, ascorbate oxidase activity in the pumpkin callus grown in the presence of 10 μM $CuSO_4$ was about 10 times that grown in the presence of 0.1 μM $CuSO_4$. In other words, ascorbate oxidase activity in pumpkin callus was markedly increased by adding copper, a prosthetic metal of the enzyme. The amount of ascorbate oxidase protein could be determined by single radial immunodiffusion method described by Mancini *et al.* (10). There was a linear correlation between the square of the diameter (D^2) of the immunoprecipitating circle and the amount of purified ascorbate oxidase added to the well (Fig. 5). Analysis with the immunological method showed that the amount of ascorbate oxidase protein increased during the growth of pumpkin callus (Fig. 6A), suggesting that the increase in ascorbate oxidase activity during the growth of pumpkin callus is accompanied by an equal increase in the enzyme protein. The increase in the amount of the enzyme protein was almost the same as that of the activity (Fig. 6A). Furthermore, the analysis showed that the amounts of ascorbate oxidase protein at 1 and 2 weeks after transfer to the fresh medium containing 10 μM $CuSO_4$ were about 5 and 8 times those in a culture containing 0.1 μM $CuSO_4$ respectively, although the increase in the ascorbate oxidase protein was a little less than that of the activity (Fig. 6). This shows that the marked increase in ascorbate oxidase activity by adding copper is also accompanied by an increase in the enzyme protein. In the present study, however, it is not yet known whether copper enhances the *de novo* synthesis of the enzyme or prevents the proteolytic breakdown of the enzyme. Keyhani and Keyhani (7) have reported that Cyt *c* oxidase activity in a *Candida utilis* yeast cells increases in response to increased copper concentration in the medium, and Shatzman and Kosman (16) have also reported that galactose oxidase activity in the fungus *Dactylium dendroides* depends on copper concentration. However, the amounts of apoproteins of these enzymes were not controlled by the copper concentration. They have suggested that copper may control the holoenzyme assembly. On the other hand, Delhalze *et al.* (3) have reported that the synthesis of apoprotein of diamine oxidase in clover leaves is regulated by the copper of the leaves so that no apoprotein is synthesized in copper-deficient leaves. Recently, Merchant and Bogorad (11) indicated that the copper-dependent expression of Cyt *c*-552 synthesis in *Chlamydomonas reinhardtii* was affected directly by the concentration of copper. In the present study, we also showed that the amount of ascorbate oxidase protein in pumpkin callus was controlled by copper, a prosthetic metal of

the enzyme. The mechanism of metal mediated regulation of protein biosynthesis is of general interest. Deikman and Jones (2) have suggested that the amount of mRNA coding for B group α-amylases in aleurone layers of barley was not affected by calcium, although the amount of the enzyme protein is controlled by the metal cofactor. They have proposed that calcium exerted its control at translation or at a site after translation. Further study will be required to determine the site where copper controls the amount of ascorbate oxidase protein in pumpkin callus.

CONCLUSION

The present study with a specific antibody against purified ascorbate oxidase showed that the increase in ascorbate oxidase activity during the growth of pumpkin callus was accompanied by an equal increase in enzyme protein. Furthermore, the marked increase in the enzyme activity by adding copper to the culture medium was also accompanied by an increase in enzyme protein. It remains to be established whether copper enhances the *de novo* biosynthesis of the enzyme or prevents its degradation.

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