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STIMULATION OF ASCORBATE OXIDASE SECRETION FROM CULTURED PUMPKIN CELLS BY DIVALENT CATIONS

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Abstract—Ascorbate oxidase is actively secreted during the growth of cultured pumpkin (*Cucurbita* spp) cells. Calcium ions markedly stimulated the accumulation of ascorbate oxidase in the culture medium. Ascorbate oxidase activity in the culture medium at 20 days after transfer to a fresh medium containing 40 mM calcium chloride ($CaCl_2$) was about 50 times the level attained in the absence of $CaCl_2$. Furthermore, the specific activity of the enzyme in the culture medium was increased *ca* 20-fold by adding 40 mM $CaCl_2$. On the other hand, Ca^{2+} had little effect on the accumulation of peroxidase in the medium. Magnesium ions, but not K^+, was shown to be effective in the stimulation of ascorbate oxidase secretion, suggesting that Mg^{2+} can substitute for Ca^{2+} in stimulating the secretion of ascorbate oxidase.

INTRODUCTION

The roles of divalent cations in cell function and regulation have been widely studied [1, 2]. In particular, calcium is known to play an important role in the secretion of proteins in various animal cells such as the pancreatic cells [3, 4], and the requirement of the intracellular elevation of calcium for exocytosis has been firmly established. Recently, much attention has been focused on elucidating the mechanism of the cell regulation by Ca^{2+} at the molecular level. Calcium has also been implicated in the secretion of proteins by plant cells. For example, Chrispeels and Varner [5], and Jacobsen *et al.* [6] reported that, in barley aleurone layers, the secretion of several enzymes including α-amylase is markedly influenced by Ca^{2+}. Mitsui *et al* [7] reported that the secretion of α-amylase by rice seed scutellar epithelium is regulated by the cytoplasmic Ca^{2+} concentration. Furthermore, Jones and Jocobsen [8] reported that Ca^{2+} increases the amount of one particular group of α-amylase isozymes secreted into the incubation medium, and Deikman and Jones [9] suggested that the presence of Ca^{2+} is necessary either for the translation of α-amylase mRNA *in vivo* or for the post-translational processing and transport of α-amylase isozymes in barley aleurone cells.

Ascorbate oxidase (EC 1.10.3.3) occurs in plant tissues such as pumpkin [10], cucumber [11] and orange [12]. The enzyme contains copper as a prosthetic metal and plays a role in secondary metabolism. The enzyme is a valuable reagent for clinical and food analyses of L-ascorbic acid [13, 14], although its precise biological function has not been clarified. We have shown that ascorbate oxidase activity in cultured pumpkin cells can be markedly increased by adding copper, a prosthetic metal of the enzyme [15], and that this increase is accompanied by an increase in the amount of the enzyme [16]. Furthermore, we found that the enzyme is secreted into the culture medium in cell suspension cultures [17]. We now report that calcium and magnesium chloride ($CaCl_2$ and $MgCl_2$) markedly stimulate the secretion of ascorbate oxidase in cultured pumpkin cells.

RESULTS AND DISCUSSION

Figure 1 shows the changes in the activities of ascorbate oxidase and peroxidase, another secreted enzyme, in the culture medium during the growth of pumpkin cells cultured in the presence of 0, 5, 10, 20, 40 and 80 mM $CaCl_2$. On adding $CaCl_2$ to the medium, ascorbate oxidase activity in the culture medium markedly increased. The activities in the culture medium after growing the cells in the presence of 40 mM $CaCl_2$ for 10 and 20 days were *ca* 10 and 50 times higher than those in the absence of $CaCl_2$, respectively (Fig. 1A). On the other hand, peroxidase activities in the medium after growing the cells in the presence of 40 mM $CaCl_2$ for 10 and 20 days were only 1.7 and 4 times those in the absence of $CaCl_2$, respectively (Fig. 1B). The amount of total protein in the culture medium was also increased by adding $CaCl_2$ (Table 1). However, the specific activity of ascorbate oxidase in the culture medium was increased by adding $CaCl_2$, since the increase in ascorbate oxidase activity in the culture medium was much more than that of total protein. The specific activities of ascorbate oxidase in the culture medium in the presence of 20 and 40 mM $CaCl_2$ were *ca* 25 and 22 times those in the absence of $CaCl_2$, respectively (Table 1). On the other hand, the specific activity of peroxidase in the culture medium was increased little by adding $CaCl_2$ (Table 1). These results suggest that the marked increase in ascorbate oxidase activity in the culture medium brought about by adding $CaCl_2$ is not due to the increase in protein washed from the cell wall surface by the increase in ionic strength, but may be due to the stimulation of ascorbate oxidase secretion.

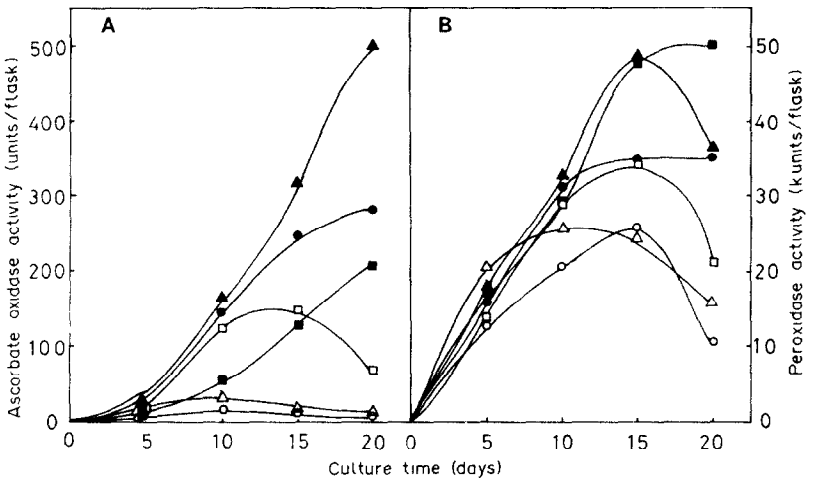

Fig 1 Changes in the activities of ascorbate oxidase (A) and peroxidase (B) in the culture medium during growth of pumpkin cell suspension cultures in the presence of various concentrations of $CaCl_2$ Pumpkin cells were inoculated into Murashige and Skoog's liquid medium containing 10 μM $CuSO_4$ with 0 (○), 5 (△), 10 (□), 20 (●), 40 (▲) or 80 mM $CaCl_2$ (■), and cultured at 25

Table 1 Effect of the $CaCl_2$ concentration of the culture medium on the specific activities of ascorbate oxidase and peroxidase in the culture medium of pumpkin cell suspension cultures

$CaCl_2$ (mM)	Protein (mg)	Ascorbate oxidase (units/mg)	Peroxidase (units/mg)
0	3 83	1 87	2500
5	5 70	2 94	2740
10	6 27	10 7	3480
20	5 99	47 7	6210
40	13 2	41 1	2950
80	9 58	24 4	5570

Cultures grown for 20 days

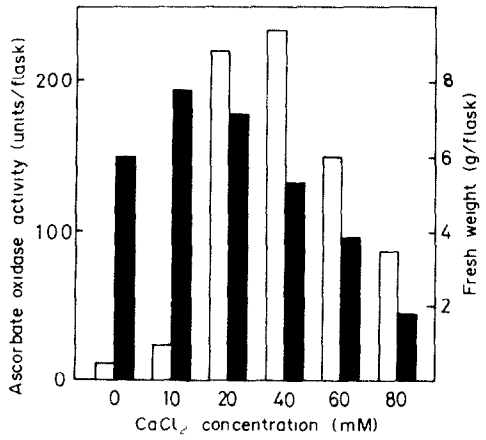

Fig 2 Effect of $CaCl_2$ concentration in the medium on ascorbate oxidase activity in the medium and fresh weight of pumpkin cells after suspension culture for 15 days □, ascorbate oxidase activity in the medium, ■, fresh weight

The effect of $CaCl_2$ on the growth of the pumpkin cells was also investigated (Fig 2) In the presence of 80 mM $CaCl_2$, cell growth was significantly inhibited, so that the ascorbate oxidase activity in the culture medium was relatively low At the other concentrations tested there was no relationship between cell growth and ascorbate oxidase activity in the culture medium, suggesting that the stimulation of ascorbate oxidase secretion by $CaCl_2$ was not the result of the effect of calcium on cell growth

Ascorbate oxidase activity in the culture medium was compared with that in the cells (Table 2) Ascorbate oxidase activity in the cells was hardly affected by $CaCl_2$ concentration, whereas that in the culture medium was markedly increased by adding $CaCl_2$ Thus the total ascorbate oxidase activity in a flask was increased by adding $CaCl_2$ (Table 2) Therefore, we infer that the synthesis of ascorbate oxidase may be also stimulated by adding $CaCl_2$ Two inhibitors (La^{3+} and ruthenium red) which affect Ca^{2+} mediated processes, were shown to have little effect on ascorbate oxidase secretion (data not shown) The effect of Ca^{2+} on ascorbate oxidase secretion may not be mediated via intracellular Ca^{2+} changes,

Table 2 Effect of the $CaCl_2$ concentration of the culture medium on ascorbate oxidase activities in the cells and in the culture medium of pumpkin cell suspension cultures

$CaCl_2$ (mM)	Cells (units/flask)	Medium (units/flask)	Total (units/flask)
0	11 9	33 1	45 0
10	13 2	38 0	51 2
20	5 01	152	157
30	11 1	229	240
40	5 98	192	198
50	4 47	166	170
60	12 0	154	166
80	6 54	102	109

Cultures grown for 15 days

although the study using selective inhibitors is not very reliable. It must be realized that there are two steps in the overall extracellular secretory process One is the secretion of the proteins from the plasma membrane and the other is the release of the proteins through the cell wall. In the case of α-amylase in cereal aleurone layers, Varner and Mense [18] concluded that Ca^{2+} and other cations facilitated the passage of α-amylase through the aleurone cell wall. On the other hand, Moll and Jones [19] concluded that Ca^{2+} influenced the transport of α-amylase molecules across the plasma membrane. Further study is required to show whether Ca^{2+} is involved in the secretion of ascorbate oxidase across the plasma membrane, or in its release to the culture medium across the cell wall

Table 3 shows the effects of $MgCl_2$ and KCl on ascorbate oxidase secretion Ascorbate oxidase activity in the culture medium was increased on adding $MgCl_2$, while the peroxidase activity in the culture medium showed a tendency to decrease on adding $MgCl_2$. When the effect of KCl was investigated, ascorbate oxidase activities in the culture medium in the presence of 10, 20 and 40 mM KCl were almost the same as those in the absence of KCl. In the presence of 80 mM KCl, ascorbate oxidase in the medium was *ca* 3 times higher than that in the absence of KCl. However, the increase may not be specific for ascorbate oxidase, since peroxidase activity in the medium was also increased by adding 80 mM KCl. Ascorbate oxidase activity in the culture medium during the growth of cells cultured in the presence of 40 mM $MgCl_2$ was compared with that in the presence of 40 mM $CaCl_2$. The stimulatory effect on ascorbate oxidase secretion of 40 mM $MgCl_2$ was almost the same as that of 40 mM $CaCl_2$ (Fig. 3), suggesting that $MgCl_2$ can substitute for $CaCl_2$ in stimulating the secretion of ascorbate oxidase.

We have shown that ascorbate oxidase secretion in cultured pumpkin cells is stimulated by adding $CaCl_2$ or $MgCl_2$, but not by adding KCl. It cannot be denied that the stimulation effect is non-specific on membrane permeability, since the effect required a high concentration of $CaCl_2$ or $MgCl_2$ However, we infer that the stimulation effect is specific for ascorbate oxidase secretion, as the specific activity of ascorbate oxidase in the culture medium is markedly increased by adding $CaCl_2$ or $MgCl_2$ (Tables 1 and 3) and the peroxidase activity in the culture medium is little increased by adding these cations (Fig. 1 and Table 3). Furthermore, there is a possibility that the stimulation effect is due to the release of ascorbate oxidase from cells which have been damaged by adding high concentration of $CaCl_2$ or $MgCl_2$. However, the observation that cell growth is not inhibited by adding 10 or 20 mM $CaCl_2$ (or $MgCl_2$), i.e. at concentrations which are effective in stimulating ascorbate oxidase secretion (Fig. 2 and Table 3), and that the total ascorbate oxidase activity in a flask is increased by adding these cations (Table 2) suggest that the stimulation effect is not due to release of the enzyme from the damaged cells. In addition, there is no denying the possibility that the stress which is caused by adding high concentration of $CaCl_2$ or $MgCl_2$ stimulates ascorbate oxidase secretion If so, ascorbate oxidase can be regarded as a stress protein such as the pathogenesis-related proteins. Further studies will be required to investigate why these divalent cations stimulate ascorbate oxidase secretion

EXPERIMENTAL

Plant material and culture methods. Pumpkin (*Cucurbita* spp., 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. [15] Disks (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 disks/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° in the dark, and the cultures were maintained by transferring *ca* 1 g of the callus every 4-weeks. Suspension cultures were established by inoculating callus cells, which had been subcultured at 4-week intervals for more than one year, into a 200 ml Erlenmeyer flask containing 50 ml of Murashige and Skoog's liquid medium with 1.0 mg/1, 2,4-D, 0.1 mg/l kinetin, 3% sucrose and 10 μM $CuSO_4$. These cultures were agitated on a rotary shaker at 100 rpm at 25°, and maintained by transferring *ca* 1 g of the cells to fresh liquid medium at *ca* 4 week intervals

Preparation of cell homogenate. The cultured cells were sepd from the suspension cultures by filtering the medium through

Table 3 Effects of the $MgCl_2$ and KCl concentrations of the culture medium on the specific activities of ascorbate oxidase and peroxidase in the culture medium of pumpkin cell suspension cultures

$MgCl_2$ (mM)	KCl (mM)	Growth rate (—)	Protein (mg)	Ascorbate oxidase (units/mg)	Peroxidase (units/mg)
—	—	3 7	8 21	1 68	2790
10	—	3 5	8 40	4 79	2130
20	—	3 7	12 9	17 1	793
40	—	3 3	16 3	20 0	541
80	—	2 1	10 5	14 2	343
—	10	3 8	7 91	1 63	2250
—	20	4 2	9 72	1 83	1860
—	40	4 0	10 9	1 21	1860
—	80	3 4	12 9	3 27	2560

Cultures grown for 15 days

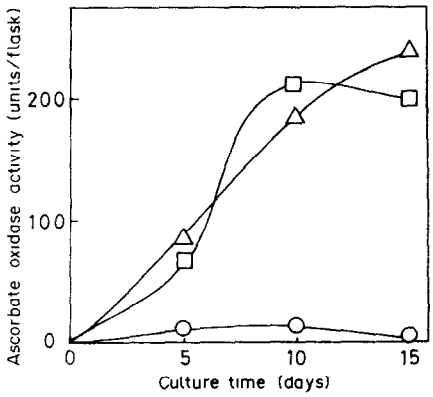

Fig 3 Changes in ascorbate oxidase activity in the culture medium during growth of pumpkin cell suspension cultures in the presence of 40 mM $CaCl_2$ and 40 mM $MgCl_2$ Pumpkin cells were inoculated into Murashige and Skoog's liquid medium containing 10 μM $CuSO_4$ without (○) and with 40 mM $CaCl_2$ (△) or 40 mM $MgCl_2$ (□), and cultured at 25°

nylon mesh (Kyoushin Ricou Co) and homogenized in 0.05 M Tris–HCl buffer, pH 7 0, with a glass homogenizer. The homogenates thus prepd were squeezed through nylon gauze and filtered through a Toyo No 2 filter. The filtrates were used as cell homogenate All the procedures were performed at *ca* 4°.

Assays Ascorbate oxidase activity was assayed at 25° by following the decrease in A_{265} of a reaction mixture containing 0 05 M K-Pi buffer (pH 7 0), 0.5 mM EDTA, 0 002% metaphosphoric acid, 0 15 mM L-ascorbic acid and enzyme soln in a final vol of 3 0 ml, according to the method of ref [20] Peroxidase activity was assayed by the method of ref [21] One unit of enzyme activity was defined as the amount of enzyme which converts 1 0 μmol of substrate per min. Protein was determined by the method of ref [22], with bovine serum albumin as the standard, after pptn with trichloroacetic acid

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