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Effect of polyamine and auxin on β–1,4-endoglucanase

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生體生長에 관한 細胞生物學的 研究
Polyamine과 auxin이 β--1,4-endoglucanase 활성에 미치는 영향

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ABSTRACT

Spermidine, spermine and IAA promoted an increase in β–1,4-endoglucanase activity in hypocotyls of *Glycine max*. The optimal concentration for the increase of the enzyme activity was 10^{-6} M for spermidine, 10^{-8} M for spermine and 10^{-6} M for IAA. However, IAA had innocuous effect on arginine decarboxylase and ornithine decarboxylase activities, and the content of polyamine. Such cumulative results suggest that the increase in β–1, 4-endoglucanase activity by IAA is not attributed by the effect on the biosynthesis of polyamine by IAA but spermidine, spermine and IAA induce cell wall loosening and therefore extension growth of cells.

Abbreviations: **IAA**: Indole acetic acid, **ABA**; Abscisic acid, **GA₃**; Gibberellic acid, **ADC**; Arginine decarboxylase, **ODC**; Ornithine decarboxylase, **Put**; Putrescine, **Spd**; Spermidine, **Spm**; Spermine, **HEPES**; N-2 Hydroxyethy-lpiperazine-N -ethanesulphonic acid, **DTT**; Dithiothreitol, **EDTA**; Ethylenediamine tetraacetate, **PMSF**; Phenylethanesulphonyl fluoride.

INTRODUCTION

Polyamine, as well its biosynthetic enzymes and precursors, is ubiquitous in all living organisms and its role in plant growth and development has been documented (Slocum *et al.*, 1984). Pol mine is known to regulate a wide variety of physiological and biochemical processes in living organisms. Some of these include regulation of cell division (Cohen *et al.*, 1984; Kaur-Sawhney *et al.*, 1980) and the activities of several enzymes involved in nucleic acid metabolism (Guilfoyle and Hanson, 1973; Wickner *et al.*, 1973; Poso and Kuosman, 1983;

Galston et al., 1978) and nuclear kinase (Datta et al., 1986; Neeraj et al., 1987). The activity of most of these enzymes is known to be controlled via some as yet unclear mechanisms by polyamine, levels of which are known to be enhanced or decreased by plant hormones via an increased or decreased biosynthesis of arginine decarboxylase, ornithine decarboxylase, diamine oxidase and polyamine oxidase, respectively. Some of these plant hormones include IAA, ABA, and GA3. IAA is also known to promote an increase in the specific activities of the enzymes involved in autolysis, mainly α-galactosidase (Sera et al., 1988). In addition, degradation of arabinogalactans and depolymerization of xyloglucans known to be plant cell wall polysaccharides appear to be involved in the mechanism by which IAA induces cell wall loosening and therefore extension growth of cells (Nishitani and Masuda, 1981).

We have already shown that polyamine enhances enzymatic activities such as β-glucan synthetase (Cho et al., 1985; Lee et al., 1987) and D-glucose-6-phosphate cyclohydrolase (Cho et al., 1986; Lee et al., 1987) known to be involved in biosynthesis of plant cell wall polysaccharides. According to previovus results (Koyama et al., 1982) high level of β-1, 4-endoglucanase in the elongating regions suggests that glucan, including xyloglucan, is hydrolyzed by the enzyme into fragments and may contribute to its loosening and ultimately to cell elongation. Given the above results, we decided to test the effect of polyamine and IAA on β-1,4-endoglucanase activity in intact Glycine max.

In the present paper we show that spermidine, spermine and IAA enhance β-1,4-endoglucanase activity, respectively. We also present evidence that the enhancement of the enzyme activity by IAA is not due to increase in polyamine level since there is no drastic change in polyamine level after the IAA treatment.

MATERIALS AND METHODS

Plant material.   Seeds of soybean (Glycine max) were grown in darkness at 23°C after soaking for 24 h. Samples were taken from 5 day old seedlings. The seedlings were treated by spray with water containing polyamine ($10^{-8} \sim 10^{-2}$M) or IAA ($10^{-8} \sim 10^{-4}$M) for 5 days.

Polyamine determination.   Methods of polyamine extraction and determination were carried out as previously described by Goren et al.(1982). Tissues were extracted in 5% cold HClO₄ at a ratio of about 200 mg/ml HClO₄. The extracts were centrifuged at 12,000 $\times$ g for 10 min and the supernatant referred to as the free polyamine fraction (20 μl of HClO₄ extract) were mixed with 400 μl of dansyl chloride (5 mg/ml in acetone) and 200 μl of saturated sodium carbonate were added. After brief vortexing, the mixture was incubated in darkness at 60°C for 1 h. Dansyl polyamines were extracted in 0.5 ml benzene and vortexed for 30 s. TLC was performed on high resolution silica gel 60 plate. Dansyl polyamine bands were photographed under UV light using polarloid 665 positive/negative film. Negativities were scanned at 595 nm in Densitometer.

ADC and ODC extraction.   ADC and ODC extraction were carried out as previously

described by Lin (1984). One gram of sample was homogenized with a glass bead in two volumes of a buffer containing 5×10^{-2} M HEPES (pH 7.4), 5×10^{-3} M DTT, 1×10^{-5} M pyridoxal-5-phosphate, 1×10^{-3} M PMSF and 5×10^{-3} M EDTA. The extracts were centrifuged at 12,000 $\times$ g for 20 min and the supernatants are hereafter referred to as ADC and ODC crude enzymes.

ADC and ODC assay. ADC and ODC assay were carried out as previously described by Lin (1984). The reaction mixture contained 2.5×10^{-2} M HEPES (pH 7.4), 2.5×10^{-3} M DTT, 5×10^{-6} M pyridoxal-5-phosphate, 2.2×10^{-4} M L-[U-C^{14}]arginine or 1.1×10^{-3} M L-[1-C^{14}] ornithine and 0.2 ml of enzyme solution. The Warburg flask was capped with rubber stopper fitted with center well containing 0.2 ml of hyamine hydroxide solution. The reaction was allowed to proceed for 60 min at 37℃ with shaking, the center well was then removed and placed in vial with 10 ml toluene-base scintillation fluid. ADC and ODC activities were presented as nmol CO_2 per g fresh weight or mg protein.

β-1.4-endoglucanase preparation. Enzymes were extracted from the wall of the *Glycine max* seedlings using the procedure of Koyama *et al.* (1980). One gram of hypocotyl was homogenized with sea sand in two volumes of 2×10^{-2} M sodium phosphate buffer (pH 6.2) containing 1×10^{-3} M EDTA, 5% glycerol, 0.01% sodium azide and 0.4 M sucrose (buffer A). The homogenate was centrifuged for 10 min at 1,000 $\times$ g. The supernatant was removed and the pellet was washed twice with the same buffer. The pellet was further homogenized in one volume of buffer A containing 1.0 M NaCl. The homogenate was centrifuged for 10 min at 10,000 $\times$ g and the precipitate was removed. The supernatant was used as a enzyme source.

β-1, 4-endoglucanase assay. 1. Assay by reducing ends: The β-1,4-endoglucanase activity was assayed with a reaction mixture containing 200 μl of 2×10^{-2} M sodium phosphate buffer (pH 6.2), 0.7% carboxymetylcellulose and the enzyme preparation in a total volume of 500 μl. This was incubated for 12~18 h at 30℃ and boiled with 500 μl of color reagent (1 g 2.5-dinitrosalicylic acid in 20 ml NaOH, 2 mol/l, 30 g K-Na tartarate in 80 ml H_2O) for 10 min at 100℃. After 30 min at room temperature, the absorbance of this sample was read at 546 nm against a blank in which the reaction mixture was added after the addition of color reagent and the glucose content obtained as reducing equivalents from a glucose standard curve.
2. Assay by viscosity: 2×10^{-2} M sodium phosphate buffer (pH 6.2), 0.7% carboxymethylcellulose and the enzyme preparation in a total volume of 8.0 ml. The incubation was carried out at 30℃ for 10 min.

Wall preparation. The cell walls were prepared using the procedure of Huber and Nevins (1979) with slight modification. Hypocotyls were homogenized in ice-cold sodium phosphate buffer (5~10 ml, 2×10^{-2} M, pH 6.2) with glass bead. The homogenate was centrifuged for 10 min at 1,000 $\times$ g. The precipitate, cell wall, was washed twice with ice-cold homogenizing buffer.

Autolysis experiments. Cell walls were suspended in 80 ml of 2×10^{-2} M sodium phosphate buffer (pH 6.2) with a few drops of toluene to avoid bacterial contamination. Autolysis

reactions were carried out in a shaker at 30℃ subsamples of 1.0 ml were at different intervals throughout the incubation time. These were centrifuged for 10 min at 12,000 × g and the cell wall residue was washed with a small amount of 2×10^{-2} M sodium phosphate buffer (pH 6.2) and replaced in the incubation medium. Upon examination of the incubation medium no contamination by bacteria or fungi was observed. The supernatant and washing were combined and the amount of total sugars was determined by the phenol-sulfuric acid method (Dubois et al., 1956).

RESULTS AND DISCUSSION

Although the occurence of the enzyme activity in a wall-bound enzyme preparation already has been reported by Koyama et al. (1981) and Byrne et al. (1975), the presence of the enzyme activity in the salt-soluble fraction from Glycine max was confirmed here (data not shown). During the first 2 h of incubation, there is a rapid decrease in viscosity. Continued incubation of the reaction mixture to 4 h results in a minimal decrease in viscosity. A linear increase in reducing equivalents is produced during the first 8 h of incubation (data not shown). Increased incubation time results in a slow rise in reducing eqaivalents for the first 50 h with a slight increase after an additional 30 h of reaction time. The low production of reducing equivalents, combined with the rapid decrease in viscosity, indicates that the enzyme hydrolyzes glucosyl bonds and that there is a limited number of enzyme recognition sites within the molecule as suggested by a previous result (Hatfield and Nevins, 1987). All hydrolytic action patterns in our experiments were similar to those of other results (Byrne et al., 1975; Hatfield and Nevins, 1987). Fig. 1 shows the progress of the autolyic process, which is known to be dependent on region (Sera et al., 1988). According to the propolsals by Labrador and Nicolas (1982) and Nishitani and Masuda (1983), a greater total release of sugars for a given fresh weight is seen in the apical region, which must undergo a greater loss of rigidity to allow higher growht rates. The intensity of the autolytic process for Glycine max was observed to much higher than that of the previous result (Sera et al., 1988).

We have already shown that the activity of β-glucan synthetase II in protoplasts increases in response to the concentration of polyamine (Cho et al., 1985). And our recent experiment shows that polyamine enhances the activity of glucanase from the hypocotyl of Glycine max in vitro (data not shown). In order to determine the effect of polyamine (10^{-8} M ~ 10^{-2} M) on β -1, 4-endoglucanase activity in intact hypocotyls, we first simplified the concentrations by eliminating 10^{-7}, 10^{-5} and 10^{-3} M of different polyamines and obtained the hypocotyls after polyamine treatment for 5 days. As shown in Fig. 2, spermine and spermidine were better stimulators than putrescine, which was not effective one. The maximum enzymatic activities were attained with concentration of 10^{-6} M of spermidine and 10^{-8} M of spermine. The patterns of enhancement of the enzyme by the polyamines are different from that of a previous report (Cho et al., 1985), in which increase in β-glucan synthetase acitivity was dependent on
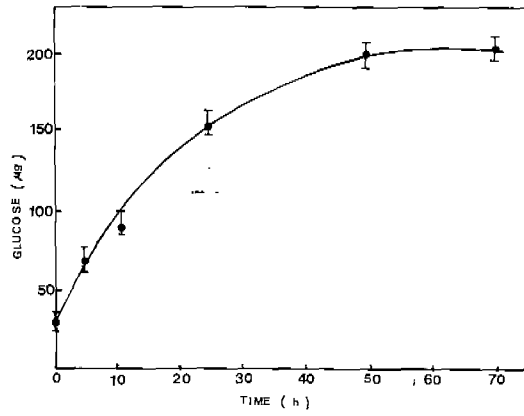
Fig. 1. Total sugars released from autolytic activity of cell walls isolated from hypocotyl of *Glycine max* cultured for 5 days as a function of time.
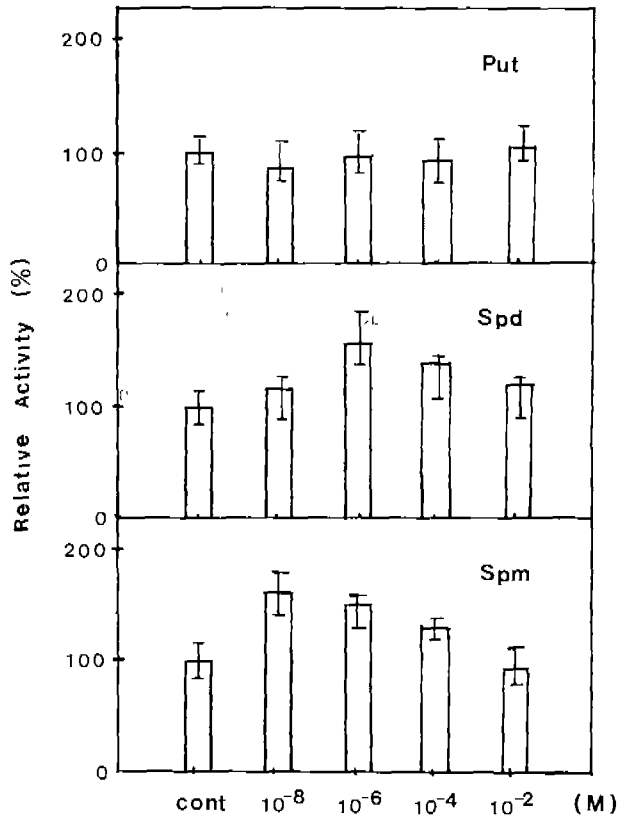

Fig. 2. Effect of polyamine on β-1,4-endoglucanase activity from hypocotyl of *Glycine max* for 5 days.
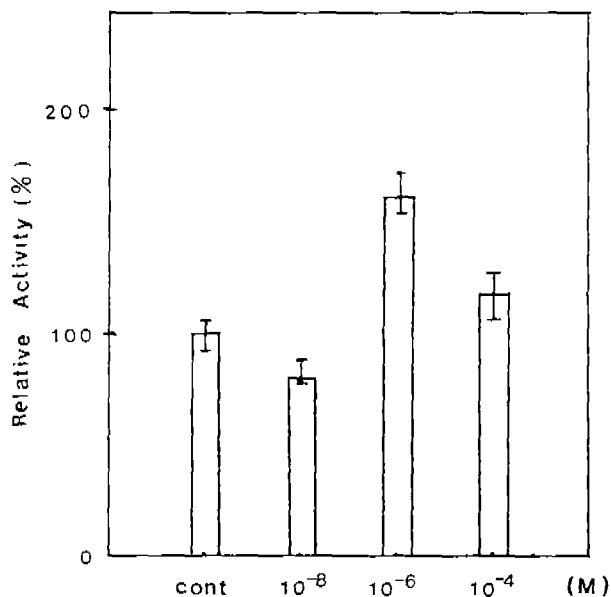
Fig. 3. Effect of IAA on β-1,4-endoglucanase activity from hypocotyl of *Glycine max* cultured for 5 days.

increased concentration of polyamine regardless of types of polyamine.

IAA has been known to increase the autolytic capacity of the cell walls by 50% via promoting an increase in the specific activities of the enzymes involved in autolysis, α-D-Galactosidase, β-D-galactosidase, β-D-glucosidase and α-L-arabinosidase (Sera *et al.*, 1988). Treatment with IAA (10^{-8} M~10^{-4} M) increased the amount of specific activities as shown in Fig. 3. The increase at 10^{-6} M was clearly dependent on time (data not shown). Also, IAA has been known to induce the activities of ornithine decarboxylase in barley seeds (Kyriakidis, 1983) and arginine decarboxylase in rice embryo (Choudhuri and Ghosh, 1982) which are known to be involved in biosynthesis of polyamine. But there was no increase in the activity of either enzyme after treatment with IAA for 5 days as shown in Tables 1 and 2. Accordingly, there was no increase in level of polyamine as shown in Table 3. According to a recent report (Kang *et al.*, 1988), IAA decreased putrescine and polyamine oxidase activities, to a point which seems to be too low to have an effect on the level of polyamine tentative as the results are. Clearly, it is important to see if IAA can promote increase in β-1,4-endoglucanase activity via increase in biosynthesis of polyamine. Our cumulative results suggest that exogenous IAA and polyamine enhance β-1,4-endoglucanase activity but exogenous IAA do not drastically change endogenous polyamine level. Therefore, IAA may not enhance β-1,4-endoglucanase activity via increase in biosynthesis of polyamine.

Table 1. Effect of IAA on the activity of ADC from hypocotyl of *Glycine max* cultured for 5 days

IAA conc.(M)	ADC	
	nmol CO_2/mg protein	(%)
Control	1.34	100
10^{-8}	1.24	93
10^{-6}	1.40	104
10^{-4}	1.36	101

Table 2. Effect of IAA on the activity of ODC from hypocotyl of *Glycine max* cultured for 5 days

IAA conc.(M)	ODC	
	nmol CO_2/mg protein	(%)
Control	0.735	100
10^{-8}	0.698	95
10^{-6}	0.756	103
10^{-4}	0.702	96

Table 3. Effect of IAA on the level of polyamine (putrescine, spermidine and spermine) from hypocotyl of *Glycine max* cultured for 5 days

IAA conc.(M)	Put	Spd	Spm
		μmol/g fresh wt.	
Control	1.20	0.95	trace
10^{-8}	1.22	0.94	trace
10^{-6}	1.11	0.96	trace
10^{-4}	1.10	0.93	trace

적   요

Spermidine, spermine, IAA는 *Glycine max*의 하배죽에서 β-1,4-endoglucanase의 활성을 증가시켰다. 효소 활성 증가의 최적 농도는 각각 10^{-6} M spermidine, 10^{-8} M spermine, 10^{-6} M IAA이었다. 그러나, IAA는 arginine decarboxylase와 ornithine decarboxylase의 활성 및 polyamine함량에 거의 영향을 미치지 않았다. 이와 같은 결과들은 IAA에 의한 β-1,4-endoglucanase활성 증가가 IAA의 polyamine합성에 대한 영향에 기인하는 것은 아니며 spermidine, spermine, IAA는 각각 세포벽 이완을 유도하여 세포의 신장을 유도할 것으로 사료된다.

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