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# Cell Biological Studies on Mechanisms of Development and Differentiation

## X. Effect of Polyamines on Glucan Synthetase Activity

**Cho, Young Dong, Sun Hi Lee*, Myung Won Kim** and Hyun Mo Lee***

(Department of Biochemistry, *Department of Biology, Yonsei University, Seoul., and **Department of Biology, Yonsei University, Wonju)

## 生體 發生 및 分化機構의 細胞生物學的 硏究

## X. Polyamine이 Glucan Synthetase 活性에 미치는 影響

趙暎東 · 李舜熙* · 金明苑** · 李賢模*

(延世大學校 理科大學 生化學科, *生物學科 · **延世大學校 原州分校 生物學科)

## ABSTRACT

The activity of *Daucus carota* L. root β-gluean synthetase II was observed to increase in the presence of polyamines such as putrescine, spermidine and spermine ***in vitro***, whereas the activity of *Daucus carota* L. root β-glucan synthetase I was not affected by the polyamine. The activity of β-glucan synthetase II from *Daucus carota* L. root protoplasts cultured on medium containing $10^{-6}$ M polyamines such as putrescine, spermidine and spermine was observed to be higher than that of the control. *Daucus carota* L. root protoplasts were observed to have the activities of arginine and ornithine decarboxylases and it was noted that they could produce polyamines, which might have an effect on β-glucan synthetase II activity.

## INTRODUCTION

Cellulose, hemicellulose, pectic polysaccharide, structural protein, and lignin have been identified as the major components of the plant cell wall. These components have been discussd in recent reviews (Albersheim, 1965; Aspinall, 1970; Cleland, 1971; Lamporte, 1970; Hühlethaler, 1967; Timell, 1964 and 1965; Whisteler and Richards, 1970). Crystalline cellulose fibers are of special interest as they make up an important part of the framework of the cell walls of higher plants. Accordingly, such enzymes as β-glucan synthetase I and II (GSI and GSII) have been investigated to determine what part they

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play in the formation of cellulose molecules. GSI has been found in Golgi bodies (Van der Woude *et al.*, 1974; Walton and Ray, 1982). GSII has been found to be associated with particle bound membranes and to be responsible for the biosynthesis of cellulose (Ray, 1973a).

Polyamines such as putrescine, spermidine, and spermine are known to have an effect on enzymatic activities such as isoleucyl-tRNA synthetase (Takeda and Ohnishi, 1975), 6-phosphogluconate and glucose-6-phosphate dehydrogenases (Mita and Yasumasu, 1980; Cho *et al.*, 1984a), RNase (Altman, 1982), and protein kinase (Kuroda *et al.*, 1982; Yan and Tae, 1982). For studying the polysaccharide components in plant cell wall in connection with enzymatic activity, cell wall regeneration in isolated protoplasts offers a useful mode. A number of methods have been developed to detect the regenerated cell walls of protoplasts (Burgress and Fleming, 1974; Horine and Ruesink, 1972; Meyer and Abel, 1975; Nagata and Takebe, 1970). Biochemical analysis of suspension cultures of protoplast revealed that the regenerated cell walls were mainly composed of glucans having 1, 3- and 1, 4- linkages and that pectic substance and hemicellulose were released into the culture medium (Takeuchi and Komamine, 1978).

In the present work, attempts were made to see if polyamines affect the activity of *Daucus carota* L. root β-glucan synthetase *in vitro* and root protoplasts. The activities of arginine decarboxylase and ornithine decarboxylase which are responsible for polyamine biosynthesis of polyamines were measured in order to see if *Daucus carota* L. root protoplasts could produce polyamines, which might affect the GSII activity.

## MATERIALS AND METHODS

**Plant materials.** Two hundreds grams of carrot (*Daucus carota*) root were washed several times and sterilized using 70% ethanol. The carrot was cut into pieces of about 10×5×5 mm, each containing cambium. The pieces were sterilized with 70% ethanol for 1 min, rinsed with 10×5×5 mm, each sterilized water, sterilized with 5% sodium hypochlorite for 10 min and washed with sterilized water three times, and then used for the protoplast preparation.

**Chemicals.** Putrescine, spermidine, spermine, mannitol, sucrose, EDTA, DTT and pyridoxal 5′-phosphate were purchased from the Sigma Chemical Co. The uridine diphospho-D-[U-$^{14}$C]-glucose (specific activity, 296 mCi/mmole) was an Amersham product. L-[1-$^{14}$C]-ornithine hydrochloride (specific activity, 53 mCi/mmole) and L-[$^{14}$C(U)]-arginine hydrochloride (specific activity, 324 mCi/mmole) were obtained from New England Nuclear. Cellulase "Onozuka" R-10 and Macerozyme R-10 were products of Yakult, Japan. Other chemicals were reagent grade.

**Concentrations of polyamines.** Polyamines ($10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M, respectively) were used for β-glucan synthetase activity assay *in vitro*. Polyamines ($10^{-6}$ M, and $10^{-3}$ M,

respectively) were added to the protoplast culture medium.

**Preparation and culture of protoplasts.** The protoplast preparation was made using a modification of methods previously used (Asamizu *et al.*, 1977; Asamizu *et al.*, 1980). Under a laminar flow hood (Clemco, Model CF335), 1 g of carrot section was incubated in 10 ml of solution consisting of 0.6 M mannitol (pH 5.4), 2% cellulase "Onozuka" R-10, 1% macerozyme R-10, and 0.5% $CaCl_2$. After 5½ hrs incubation, the cell debris was removed by stainless steel sieve(100 μm), and the protoplasts formed were collected by centrifugation at 100 *g* for 5 min and washed three times with 0.6 M mannitol solution (pH 5.4) containing a basal salt culture medium. The density of protoplasts was adjusted to $2\times10^5$ protoplast/ml. The protoplasts were cultured in the dark on petri dishes 50 mm in diameter on Murashige and Skoog's medium(1962) containing 0.1 M sucrose, 0.5 M mannitol, and 4.52 μM 2,4-D. Evans blue solution containing 0.5 M mannitol (Kanai and Edward, 1973) was mixed with the protoplast preparation in the ratio of 1 to 1 in volume and the number of surviving protoplasts counted by hemocytometer.

**Preparation and assay of β-glucan synthetase.** Preparation of the enzyme and determination of the enzyme activity were done using a modification of the method used by previous workers (Cerenius and Söll, 1984; Ray, 1973b; Ray *et al.*, 1969). Briefly, segments (2~4 g) were ground in an ice-cold mortar for 5 min at 0°C in 4 ml of 50 mM Tris buffer (pH 8) containing 0.25 M sucrose, 1 mM Na-EDTA, 0.1 mM $MgCl_2$, and 10 mM KCl. The homogenate was squeezed through nylon cloth and centrifuged at 6800 *g* for 10 min. The precipitate which contained negligible synthetase activity was discarded. The supernatant was recentrifuged at 40,000 *g* for 45 min and the pellet was used as a crude enzyme. In the preparation of GSII from cultured protoplasts, the same methods were applied except for reducing the time of grinding the tissues.

The enzyme assay mixture consisted of 200 μl enzyme solution, 800 μl Tris buffer containing 0.02 μCi uridine diphospho-D-[U-$^{14}$C]-glucose, 0.5 mM cold UDP-glucose and polyamines, and this was incubated at 27°C for 1 hr. In case of GSI, 58 mM $MgCl_2$ was added to the Tris buffer, but no $MgCl_2$ was used for GSII. 1 ml of 10% TCA was added to the enzymatic reaction mixture which was then filtered through a Whatman GF/C glass filter, washed with 3 ml of 10% TCA three times, and with 3 ml of 70% ethanol three times.

After drying, it was transferred to 10 ml of scintillation fluid and counts recorded in the liquid scintillation counter (Packard Trib-Carb 4530).

**Preparation and assay of arginine and ornithine decarboxylases.** Preparation and assay were made using a modification of a previous method (Altman *et al.*, 1982). Briefly, sections were ground in a prechilled mortar with a pestle (250~600 mg/2 ml extraction medium). The extraction medium consisted of 10 mM phosphate buffer (pH 7.2), 0.1 mM DTT, 1 mM pyridoxal phosphate, and 20 mM Na-EDTA. The extract was centri-

fuged at 12,000 *g* for 20 min and the supernatant was used immediately as the enzyme source. The enzyme reaction was started by adding 200 $\mu$l crude enzyme to the reaction vessel containing 300 $\mu$l of the extraction medium and 0.5 $\mu$Ci L-[$^{14}$C(U)]-arginine or 0.5 $\mu$Ci L-[1-$^{14}$C]-ornithine. The evolved $^{14}CO_2$ was absorbed on Whatman No. 3 (20×20 mm) wet by 200 $\mu$l of 2 N KOH. The reaction was allowed to proceed for 60 min at 37°C in a shaking water bath and was terminated by injection of 5% $HClO_4$ solution. Control value was obtained by using boiled crude enzyme. The Whatman papers were removed and placed in vials for counting of the radioactivity. In case of cultured protoplasts similar methods were used.

## RESULTS AND DISCUSSION

It appears that the activity of the freshly prepared GSI was slightly enhanced by spermidine *in vitro*, whereas putrescine and spermine had no effect on the activity of the enzyme at concentrations from $10^{-3}$ M to $10^{-5}$ M (Fig. 1). But in the case of GSII, putrescine, spermidine and spermine were observed to enhance the activity *in vitro* (Fig. 2). Yan and Tao (1982) previously reported that wheat germ protein kinase was activated at low concentrations of polyamine but inhibited at high concentrations. In our preliminary experiment, polyamines ($10^{-2}$ M and $10^{-1}$ M) were observed to have an inhibitory effect on the activities of GSI and GSII *in vitro*. However, such high concentrations seem to cause problems such as change in pH of the buffer and higher than endogenous concentrations of polyamines. It is very interesting that polyamines have more effect on GSII activity than GSI, suggesting different behaviors of the two enzyme with polyamines. In addition, the enhancement of the GSII activity by polyamines as in order of tetraamine (spermine), triamine (spermidine), and diamine (putrescine). Tentative as the results are, they support the idea that the negatively charged part of GSII possibly interact with the positively charged polyamine as suggested by a previous report (Altman, 1982). In addition, there are many more reports on the effects of polyamines on animal enzymes compared to that on plants; polymerase(Tanaka, 1982), phosphatidylinositol phosphodiesterase (Eichberg *et al.*, 1981), isoleucyl-t-RNA synthetase (Igarash *et al.*, 1978), guanylate cyclase (Liebel and White, 1982), and phosphorylase C (Nahas and Graff, 1982). But the exact mechanisms of inhibition and enhancement of enzymatic activity by polyamines even in animals seem to be far from clear, though Yan and Tao suggest that polyamines can partially replace $Mg^{2+}$ in the case of wheat germ protein kinase.

Putrescine, spermidine and spermine were observed to cause a steady increase in the activity of GSII (Fig. 3). A drastic increase in enzymatic activity was observed over 3 days for the carrot protoplasts cultured on medium containing $10^{-6}$ M of polyamines. However, carrot protoplasts cultured on medium containing $10^{-3}$ M of polyamines was observed to have a lower enzymatic activity compared to that of protoplast cultured on medium

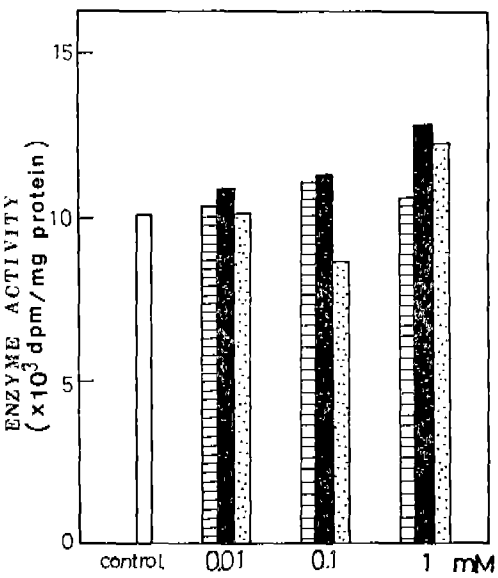


Fig. 1. Effect of putrescine, spermidine and spermine on carrot root glucan synthetase I activity *in vitro*. Polyamines were added to the enzyme assay mixture to the indicated final concentration. Enzyme activities are expressed as the amount of radioactivity measured from 0.02 $\mu$Ci UDP-[U-$^{14}$C]-glucose/mg protein after incubation with the enzyme at 27°C for 1 hr.
▤, putrescine; ■, spermidine; ▨, spermine.

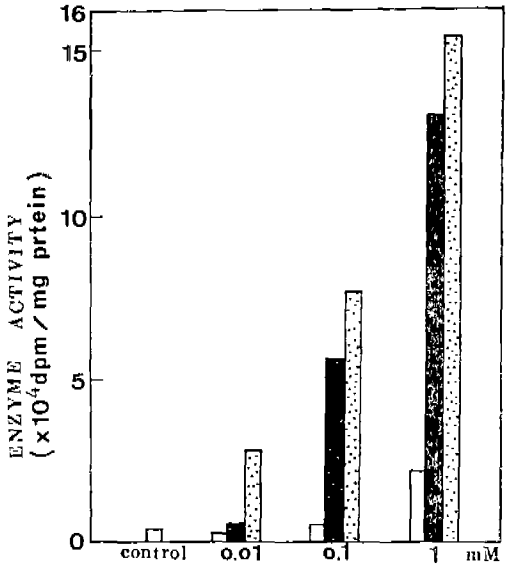


Fig. 2. Effect of putrescine, spermidine and spermine on carrot root glucan synthetase II activity *in vitro*. Polyamines were added to the enzyme assay mixture to the indicated final concentration. Details of enzyme assay are the same as in Fig. 1.
▤, putrescine; ■, spermidine; ▨, spermine.

containing $10^{-6}$ M of polyamine (Fig. 4). The GSII activities in protoplasts cultured on medium containing $10^{-4}$ M and $10^{-5}$ M of polyamines (data not shown) were found to be lower than the GSII activity in protoplast cultured on medium containing $10^{-6}$ M polyamines but higher than the GSII activity in protoplast cultured on medium containing $10^{-3}$ M polyamines. In protoplasts the polyamines may enhance enzymatic activity itself and bind to DNA, RNA and phospholipids whithin reach as claimed by a previous report (Igarashi *et al.*, 1982), and possibly enhance the biosynthesis of isoleucyl-t RNA synthetase (Igarashi *et al.*, 1978).

There is a considerable body of evidence that the aliphatic amines, putrescine, spermidine, and spermine are closely linked to vital cell functions and that a significant amount of polyamine is present in nuclei (Cohen, 1971). In addition, there is a claim that the control of nuclear protein kinase N II activity may be an essential factor in regulation of gene expression as the phosphorylation of nonhistone chromosomal protein by protein kinase N II is considered to be intimately involved in gene activation (Stein *et al.*, 1974).

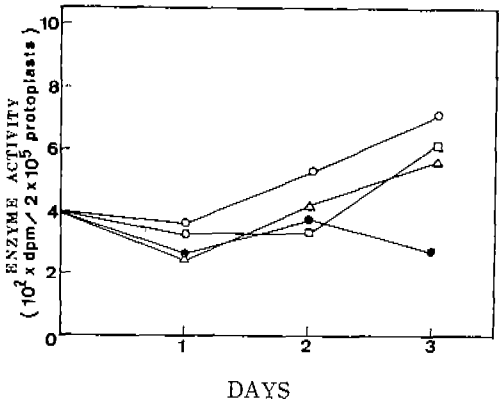


Fig. 3. Effect of putrescine, spermidine and spermine on carrot root protoplast glucan synthetase II as a function of time. Polyamines used are $10^{-6}$ M; (•), control; (○), putrescine; (△), spermidine; (□), spermine.
In these assays enzyme activities are expressed as the amount of radioactivity measured from UDP-[U-$^{14}$C]-glucose (0.02 $\mu$Ci)/$2 \times 10^5$ protoplast after incubation with 200 $\mu$l of the enzyme at 27°C for 1 hr.

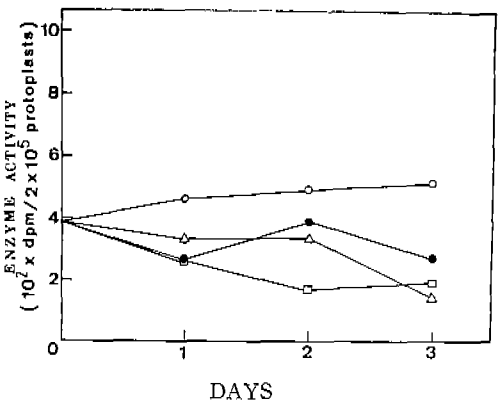


Fig. 4. Effect of putrescine, spermidine and spermine on carrot root protoplast glucan synthetase II as a function of time. Polyamines used are $10^{-3}$ M; (•), control; (○), putrescine; (△), spermidine; (□), spermine. Details of enzyme assays are the same as in Fig. 3.

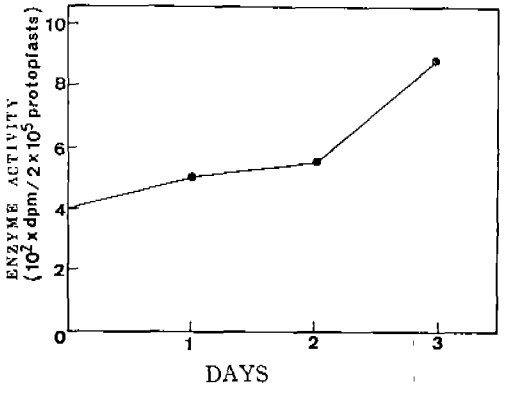


Fig. 5. Activity of arginine decarboxylase in carrot root protoplast as a function of time.
In these assays enzyme activities are expressed as the amount of radioactivity released as $^{14}CO_2$ from 0.5 $\mu$Ci L-[U-$^{14}$C]-arginine/$2 \times 10^5$ protoplasts after incubation with 200 $\mu$l of enzyme at 37°C for 1 hr.

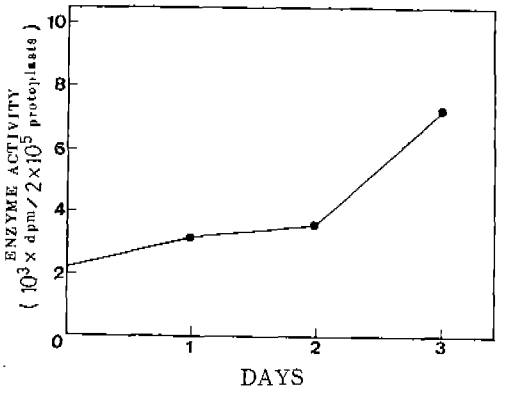


Fig. 6. Activity of oithine decarboxylase in carrot root protoplast as a function of time.
In these assays enzyme activities are expressed as the amount of radioactivity released as $^{14}CO_2$ from 0.5 $\mu$Ci L-[1-C$^{14}$[-ornithine/$2 \times 10^5$ protoplasts after incubation with 200 $\mu$l of enzyme at 37°C for 1 hr.

According to the previous report (Takeuchi and Komamine, 1981), a major component of the plant cell wall in protoplast at an early stage is β-glucan, which has been observed to begin to form netlike cellulose in protoplasts in 3 days from culture and differentiate in 4 days (Asamizu *et al.*, 1977). Considering the enhancement of β-glucan synthease II activity by polyamines ($10^{-6}$ M) in 3 day protoplasts (Fig. 3), the role of polyamine might be significant as far as β-glucan biosynthesis is concerned.

In connection with the enhancement of GSII activity in protoplast cultured on medium containing polyamines, the activities of arginine and ornithine decarboxylases responsible for polyamine biosynthesis (Altman *et al.*, 1982; Cohen *et al.*, 1982; Cho *et al.*, 1982, 1983, and 1984a) in protoplasts were checked in order to see if the two enzymes might have activities which would produce polyamines. As shown in Figs. 5 and 6, both enzymatic activities were observed to increase steadily in the protoplasts. Considering the two enzymatic activities, the two enzymes seem to produce enough endogenous polyamines to enhance GSII activity.

## 摘 要

당근(*Daucus carota* L.)뿌리의 β-glucan synthetase II의 활성은 *in vitro* putrescine, spermidine 및 spermine에 의하여 높아졌다. 반면에 β-glucan synthetase I의 활성은 putrescine, spermidine spermine에 의하여 별영향을 받지 않았다. $10^{-6}$ M의 putrescine, spermidine 및 spermine을 각각 포함하는 배지에서 배양된 당근의 root protoplasts에서 β-glucan synthase II 활성은 대조군보다 높은 경향을 보였다. 또한 원형질체는 arginine decarboxylase와 ornithine decarboxylase의 활성을 갖고 있음을 관찰하였고, 이 두 효소에 의하여 합성된 polyamines은 β-glucan synthetase II 활성에 영향을 줄 것으로 사료된다.

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