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Peroxidase Isozyme in Root Differentiation from Cultured Ginseng Root Explants

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인삼 근절편 배양시 Peroxidase Isozyme에 관한 연구

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ABSTRACT

In order to pursue some physiological studies on organogenesis in ginseng tissue culture, ginseng root explants were cultured on a modified MS medium containing NAA and kinetin. The activities of peroxidase and some enzymes were investigated and their isoenzyme patterns were also observed. The activity of peroxidase decreased by 20% in one week's culture and increased thereafter by 80% in culturing for 7 weeks compared with the control group. Glucose-6-phosphate dehydrogenase activity increased after 3 weeks' culture and 6-phosphogluconate dehydrogenase activity increased by 400% after culturing for 5 weeks and increased during the days preceeding root formation. The activities of glutamate dehydrogenase and acid phosphatase also increased during the culture. After 3 weeks' culture, new peroxidase isozyme (pH 7.6) appeared and 7 weeks' culture, another new peroxidase isozyme (pH unidentified) appeared. These patterns were also identified by using FPLC. After 7 weeks' culture, a new esterase isozyme of pH 8.5 appeared and isozyme patterns of acid phosphatase were quite changed compared with the isozyme patterns of tissue cultured for 5 weeks. In so far as these new isoenzymes appear distinctively after 7 weeks' culture, root differentiation is supposed to be induced after 7 weeks' culture.

INTRODUCTION

Plant growth and differentiation are controlled by hormones and induced by the correlation between the hormones and the enzymes involved in specific metabolism.

These enzymes are found to appear as variable isozymes according to the period of differentiation and growth (Scandalio, 1964; Siegel and Galston, 1967; Seeni and Gnanam, 1981), as well as plant organ and tissues(Yoneda, 1970; Elkinawy and Raa, 1973). The

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variations of the isozyme patterns are studied in terms of morphological changes and biochemical factors during differentiation(Scandalio, 1974).

Peroxidase, first isolated from a horseradish root in 1966, is known as one of the principal enzymes which control plant cell growth and development(Kochba *et al.*, 1977). Although much information is available on such function as oxidation of indole-3-acetic acid(Frenkel, 1972; Birecka *et al.*, 1973; Gove and Hoyle, 1975) lignification(Mäder and Füssl, 1982), oxidation of some phenolic compounds(Schafer *et al.*, 1971) and oxidation of pyridoxal-related compounds(Hill, 1970; Kim,1978), they have not been proved manifestly. It has been reported that peroxidase activity increased and a corresponding new isozyme appeared when supplemented by auxin and kinetin(Kim and Kaang, 1983). There are reports demonstrating that the isozyme pattern of peroxidase changed in embryonic orange ovular callus(Kochba *et al.*, 1977), developing endosperm of maize(Padma and Redoy, 1970), rooting hypocotyl of *I. balsamina*(Nanda and Kaur, 1973), and mungbean hypocotyl (Chandra and Worley, 1973).

Also, the principal enzymes of pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were reported to increase in their activity of the pathway related enzymes increased during the differentiation of xylem in poplars and such an increase corresponds with structural change of the xylem(Sagisaka and Asads, 1981).

It has been considered, therefore, that the increasing activity of peroxidase and other enzymes of the pentose phosphate pathway, and the newly appearing isozymes indicate plant differentiation(Wolter and Gorden, 1975; Seeni and Gnanam, 1981).

In addition to these enzymes, some others, such as glutamate dehydrogenase(Chou and Splittstoesser, 1972; Takahashi and Furuhashi, 1980), and acid phosphatase (Meyer and Harel, 1971; Suzuki and Sato, 1973, 1976), esterase (Scandalio, 1964, 1974), were reported to have an effect upon plant growth and development.

As for ginseng, its growth is slow, and culture is difficult, while its differentiation is variable(Chang and Hsing, 1980; Choi and Kim, 1981). Also various isozyme patterns of peroxidase and other enzymes mentioned above exist among the various ginseng species (Son and Park, 1984). Recently, we reported that the isozyme patterns of peroxidase changed in ginseng callus treated with 2,4-D and kinetin(Kim and Kang, 1983). We cultured ginseng root explants in a modified MS medium containing NAA and kinetin to induce the root differentiation(Choi and Kim, 1981) and investigated the enzyme activity and isozyme patterns of peroxidase, as well as several pentose phosphate pathway enzymes to show evidence for root differentiation.

MATERIALS AND METHODS

Materials. Ginseng roots were sterilized in 70% ethyl alcohol for 1 min and 7% sod-

aceton (Wetter and Dyck, 1985).

FPLC(Fast Protein Liquid Chromatography). Ginseng explants were homogenized with 50mM phosphate buffer (pH 6.0) and centrifuged at 24,000 xg for 20 min. The supernatant was subjected to 30% to 80% ammonium sulfate fractionation. The pellet dissolved in 5 mM phosphate buffer(pH 6.0) and desalted by dialysis in the same buffer for 24 hrs (Kim, 1978). The concentrated enzyme source was completely desalted by a Sephadex G-25 column and loaded in anion exchange column(Mono Q, HR 5/5, 5 x 50 mm) of FPLC (Pharmacia Fine Chemicals). Th column was eluted with linear gradient by 5 mM phosphate buffer(pH 6.0) containing 0.35 M NaCl for 15 min at 2 ml/min. The protein amount of each fraction was measured at 280 nm and the activity of peroxidase was measured to investigate the peroxidase peak(Schafer *et al.*, 1971).

IAA assay. Cultured root explants were homogenized with 50 mM phosphate buffer (pH 7.0) containing sodium ascorbate. After centrifuging at 15,000 xg for 15 min, the supernatant was applied to chromosorb P and then partitioned against hexane. The aqueous phase was eluted with MeOH. The MeOH portion was separated with HPLC, waters model 450.

RESULTS AND DICUSSION

The organogenetic behaviour of ginseng callus was quite interesting. NAA(naphthaleneacetic acid) 2.5×10^{-5}M and kinetin 4.5×10^{-6}M induced root differentiation in three months(Choi and Kim, 1981), BA(6-benzylamino purine) GA and 2 ip(Δ^2-isopentylaminopurine) favoured shoot differentiation in eight months(Chang and Hsing, 1980). So, in this study, root explants were cultured on agar solidified MS media supplemented with NAA 2.5×10^{-5}M and and kinetin 4.5×10^{-6}M for 7 weeks. Changes in peroxidase activity and isoperoxidase pattern were examined every 2 weeks.

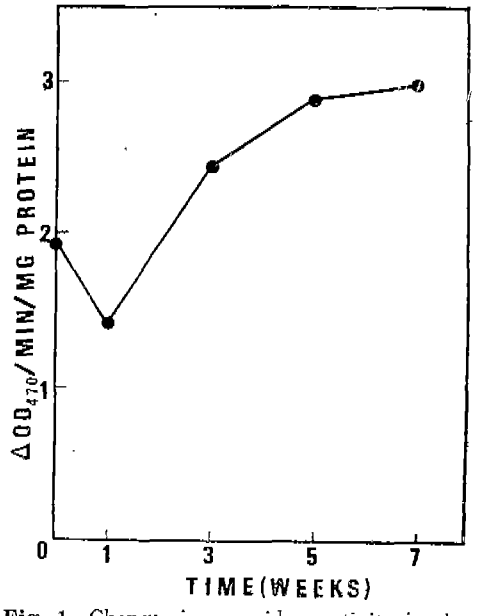


Fig. 1. Changes in peroxidase activity in ginseng root explants cultured on modified MS medium containing NAA 2.5×10^{-5} M and kinetin 4.5×10^{-6}M.

After one week in culture, the peroxidase activity decreased by 20%, when compared with unorganized ginseng root explants cultured in media without exogenous hormone but increased again during the days preceeding root formation. After culturing for 7 weeks, the peroxidase activity incre-

ium hypochlorite solution for 25 min. For root differentiation, the segments of sterilized root were cultured on a modified MS medium containing NAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M under light at 25°C every 2 weeks.

Enzyme extraction and assay. Cultured root explants were homogenized with 50mM phosphate buffer (pH 7.0) and the homogenate was centrifuged for 20 min at 24,000 xg. The resulting supernatant was used as the crude enzyme source. All operations were carried out at 4°C(Bartel and Huystee, 1984).

Peroxidase assays with respect to guaiacol as substrate were accomplished using a procedure based upon that of Schafer *et al.* (1971). Assays contained 40 mM phosphate buffer (pH 6.5), 9.5 mM guaiacol, 5 mM H_2O_2 and 50 μl crude enzyme source. The enzyme activity was measured the increase in absorbance at 470 nm by a spectrophotometer.

Glucose-6-phosphate dehydrogenase(G-6-P DH), 6-phosphogluconate dehydrogenase (6-P-G DH)and glutamate dehydrogenase(GDH) activity were determined by following the changes in absorbance at 340 nm at 30°C by the revised method of Seo and Kim(1982) and the method of Kanamori *et al.* (1972). The reaction mixture for G-6-P DH and 6-P-G DH contained 1 mM substrate 0.2 mM $NADP^+$, 0.3 mM $MgCl_2$ and 0.3 mM Tris-HCl (pH 8.0) and for GDH, 0.07 M Tris-HCl (pH 8.0), 0.03 M α-ketoglutaric acid, 0.03 M NH_4Cl, and 1 mM NADH. One unit of enyyme is defined as 0.01 change of absorbance per min.

Acid phosphatase activity was determined from absorbance at 450 nm using p-nitrophenyl phosphate as the substrate (Meyer and Harel, 1971). The reaction was started by adding crude enzyme source to buffered substrate solution (pH 5.0) preincubated for 5 mim. at 30°C. Incubation was carried out for 30 min at 30°C and terminated by adding 0.1N NaOH.

Isoelectric focusing and activity staining. Isoelectric focusing was carried out by modified method of Stegeman and Park(1979). 6% acrylamide gel with 0.1% ampholine, 200 μl crude enzyme with 10% sucrose and 0.1% ampholine solution with 5% sucrose were loaded in order to prevent mixing and were run at 100V for 1hr and at 200V for 4hrs at 4°C. 0.1% H_3PO_4 was used in the anodic solution, and saturated $Ca(OH)_2$ in the cathodic solution. After the running, we carried out activity staining to observe the isozyme patterns. And, the gel was cut at 1 cm intervals without activity staining, and extracted by distilled water in pH 7.0 to measure the gel pH gradient.

Peroxidase bands were visualized by incubating the gel in a solution of 50 mM phosphate buffer(pH 6.0) in 1% guaiacol and 0.2% H_2O_2 (Kim, 1978). Acid phosphatase was detected by Diazonium system (Suzuki and Sato, 1976). The gel was incubated in a staining solution for 30 min at 40°C after removing ampholine from the gel. Staining solution was 0.5M veronal acetate buffer (pH 5.0) which contained α-naphthyl phosphate, pararosaniline-HCl and $NaNO_2$. Esterase was dectected by incubating the gel at 35°C for 15 min in 0.1M Tris-HCl buffer(pH 7.3) containing α-naphthyl acetate, fast blue RR-salt and

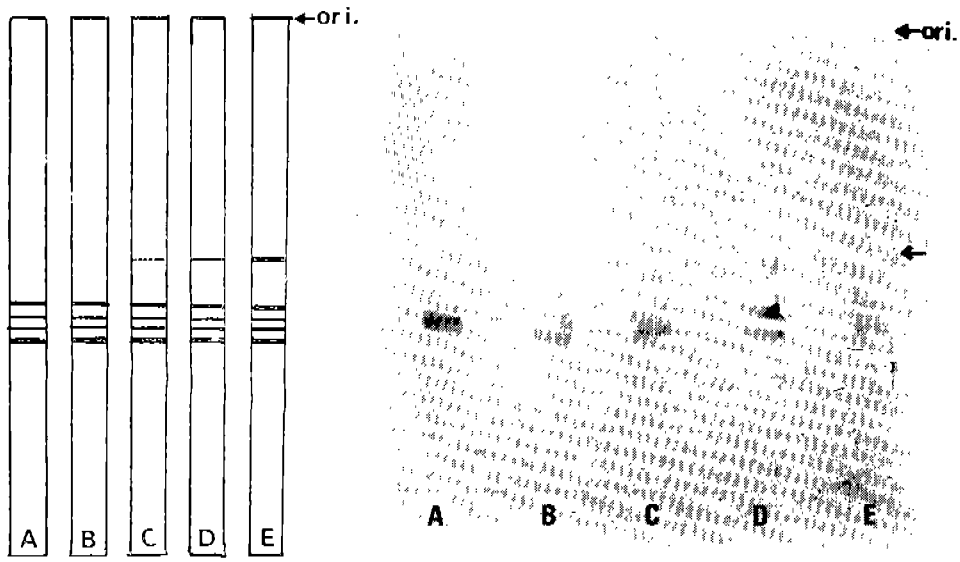


Fig. 2. Isozyme patterns of peroxidase. A, control; B, cultured for 1 week; C, 3 weeks; D, 5 weeks; E, 7 weeks.

ased by 80% compared with the control group (Fig. 1).

During the induction of plant regeneration, the plant hormone must be balanced in media which callus is culturing. Auxin especially affects the transcription of RNA in the synthesis of enzymes which regulate plant regeneration(Katsuaki, 1980). And so peroxidase activity related to IAA oxidase, and peroxidase isozyme patterns are changed during organ-formation. Cathodic peroxidases especially are considered to be involved in auxin catabolism (Gasper *et al.*, 1985), where fast-moving anodic peroxidases have been associated with lignification (Mäder and Fussel, 1982). The peroxidase activity in root-forming tissues increased, too (Gaspar *et al.*, 1985). Distinctive changes in the peroxidase isozyme patterns during rooting also occur, viz; a continuous increase in number and intensity of anodic peroxidases, and an increase in intensity for cathodic peroxidases up to a maximum followed by a decrease in intensity. These changes have been interpreted as indicating a continual process of lignification and the two phase requirement for endogenous auxin; reduction

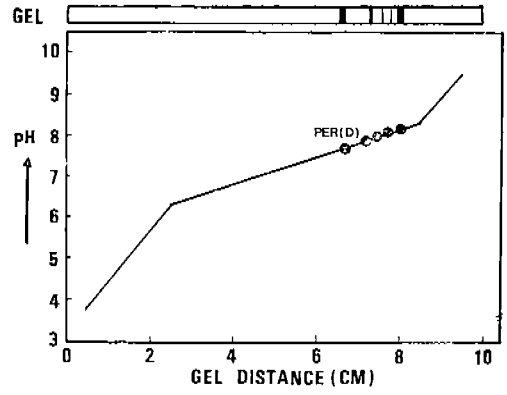


Fig. 3. Isoelectric point of peroxidase in ginseng root explants cultured on modified MS medium containing NAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M for 7 weeks. Isoelectric focusing was carried out in the tube of acrylamide gel (amp holine, pH 3~10. PERCD) is new peroxidase isozyme in 7 weeks' culture.

of auxin during root induction (i.e., the phase in which no histological events are observable) and an increase in auxin during root initiation (i.e., the phase during which root primodium formation begin) (Gaspar *et al.*, 1985).

We have shown that ginseng callus cultured in media supplemented with NAA and kinetin is capable of rooting (Choi and Kim, 1981). As a matter of fact, the intensity of peroxidase as well as the pattern of peroxidase isozyme change during root formation (Fig. 2). After culturing for 7 weeks, peroxidase activity increased by 80%, and a distinctive new peroxidase isozyme appeared (Fig. 2). Endogenous IAA content also increased at 5 weeks (Fig. 5).

The isoelectric point of the new band is pH 7.6 and we can assume that new peroxidase isozyme (pH 7.6) and appearing at 3 min, flow other new peroxidase isozymes (pH unidentified appearing after 10 minutes flow in FPLC must play an important role in root formation of ginseng tissue culture) (Figs. 3, 4).

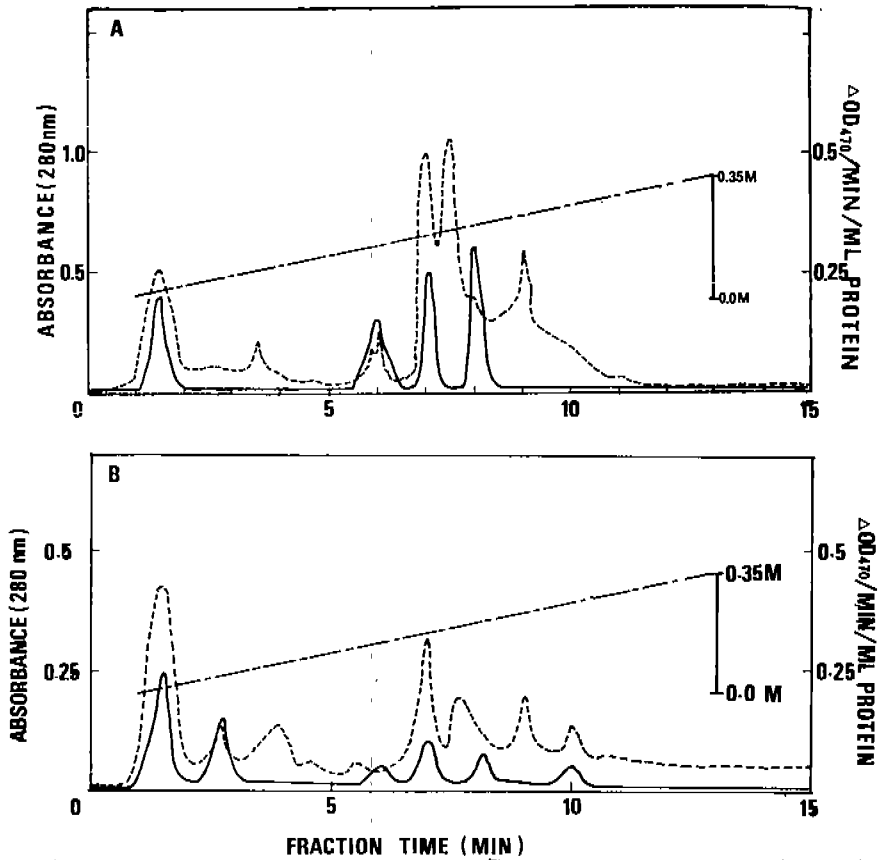


Fig. 4. Elution pattern for peroxidase in ginseng root explants by FPLC ion exchange chromatography. Elution was performed with linear salt gradient (0~0.35M) in 10 mM phosphate buffer pH 6.0). A, control; B, cultured on a modified MS medium containing NAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M for 7 weeks. Symbols: (·····), absorbance at 280nm; (—), peroxidase activity.

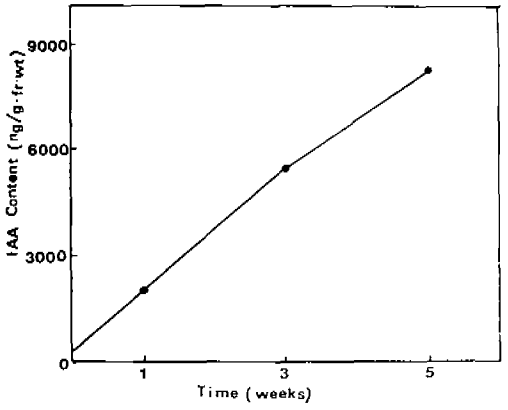


Fig. 5. Amount of endogenous IAA in ginseng root explants cultured on modified MS medium containingNAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M

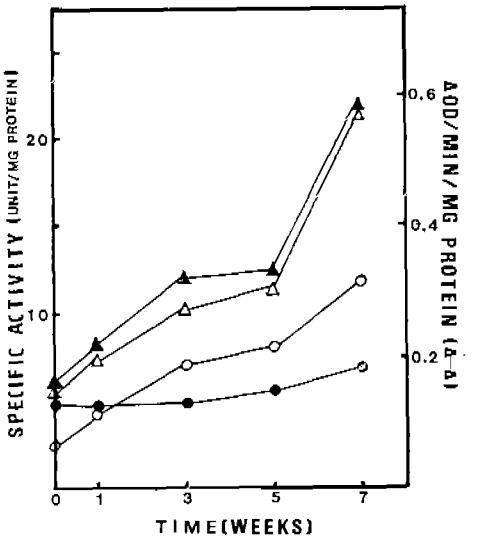
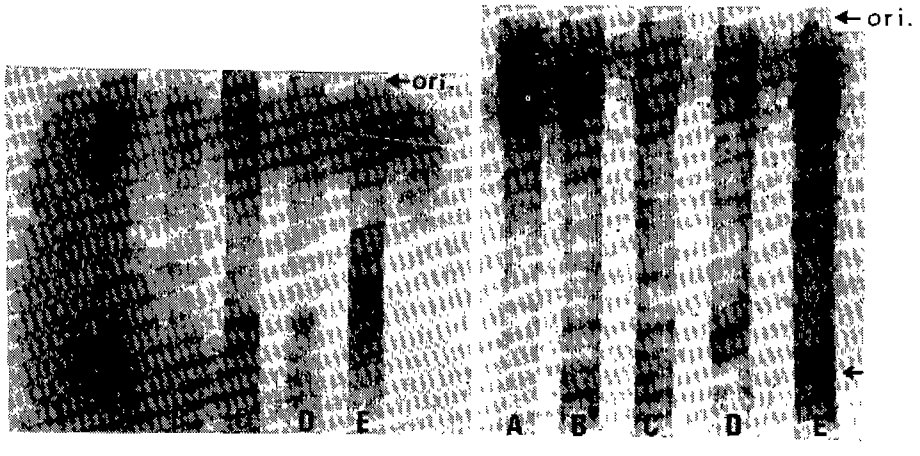


Fig. 6. Changes in enzyme activity in ginseng root explants cultured on modified MS medium containing NAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M. One unit of enzyme is defined as an absorbance of 0.01 at 339 nm per minute.
●; glucose-6-phosphate dehydrogenase,
○; 6-phosphogluconate dehydrogenase,
▲; glutamate dehydrogenase,
△; acid phosphatase.

In this study, we determined changes in the activity of several enzymes during root differentiation in cultured ginseng explants. The experiments showed that the activities of acid phosphatase, esterase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase were higher in root forming callus, while no morphological events were observable until after 7 weeks(Figs. 6, 7). Three months later, differentiated roots could be observed(Fig. 8).



Acid phosphatase Esterase

Fig. 7. Isozyme patterns. A, control; B, cultured for 1 weeks; C, 3 weeks; D, 5 weeks; E, 7 weeks.

Fig. 8. Root differentiation in ginseng root explants cultured on modified MS medium containing NAA 2.5×10^{-5}M and kinetin 4.5×10^{-6}M.

The changes in peroxidase activity reflect corresponding changes in IAA oxidase activity and a marked increase in tissue peroxidase activity just prior to shoot initiation tends to support the view that regulation of endogenuous auxin in a prelude to the onset of the developmental process. Peroxidase may also be involved in rhizogenesis, and there are distinctive peroxidase isozyme patterns that occur during root initiation. These various isozymes probably have specific roles during differentiation(Throp, 1978).

In ginseng tissue culture, there were also marked increase in peroxidase activity, new peroxidase isozyme and increase in activity of some enzymes, and also their now isoenzymes were beginning to appear by 7 weeks culture prior to root formation.

One remarkable thing is that peroxidase activity which is known to be related to IAA oxidase, and endogenous IAA were increased by ginseng tissue culture. The relations between peroxidase isozyme and endogenous auxin during organ formation in ginseng tissue culture and the specific role of peroxidase isozyme in differentiation must be identified in the future.

摘 要

인삼 조직배양에 있어서 기관분화에 대한 생리학적 연구를 위하여 인삼근절편을 NAA 2.5×10^{-5}M과 kinetin 4.4×10^{-6}M이 첨가된 MS변형배지에서 배양하였다. 식물 분화과정과 관계있는 peroxidase 및 몇가지 효소의 활성과 isozyme양상을 조사하여 다음과 같은 결과를 얻었다. Peroxidase는 배양 1주에서 20%정도 효소활성이 감소하였으나 배양기간이 경과함에 따라 대조구에 비하여 80%까지 증가하는 양상을 나타내었다. Glucose-6-phosphate dehydrogenase의 활성은 배양 3주부터 증가하기 시작하였으며, 6-phosphogluconate dehydrogenase의 활성은 배양 5주까지 400% 이상 급격히 증가하였고 뿌리분화가 유도됨에 따라 계속 활성이 증가하였다. 이외에 glutamate dehydrogenasec acid, phosphatase의 경우에도 배양기간이 경과함에 따라 효소활성이 증가하였다. 3주 배양후에 동전점 pH7.6의 새로운

isoperoxidase가 관찰되었고 7주 배양후에 또 다른 isoperoxidase가 나타났으나 이 peroxidase의 등전점은 조사하지 못하였다. 이 양상은 FPLC에 의해서 분리한 것과 일치하는 결과를 나타내었다. 또 esterase는 인삼근 절편 배양 7주후에 등전점의 pH 8.5에서 새로운 isozyme band가 관찰되었으며, acid phosphatase의 경우도 배양 5주후의 근절편에서 isozyme 양상이 현저하게 변화하였다. 새로운 isozyme이 배양 7주 후부터 특이하게 나타나는 것으로 미루어보아 배양 7주 경과후 부터 인삼근절편의 근분화가 유도되는 것으로 사료된다.

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