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Isolation of Protoplasts from Cultured Cells of Potato (*Solanum tuberosum* L.) Tuber Tissue

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감자(*Solanum tuberosum* L.) 괴경의 培養細胞로부터 原形質體의 分離

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ABSTRACT

Protoplasts were isolated from cultured cells of potato (*Solanum tuberosum* L.) tuber tissue. The ability of callus formation from the cultured cells was higher in cultivars Dejima and Superior than in Shimabara and Irish Cobbler on Lam's medium. Therefore, the former was used as sources for protoplast isolation. Friable calli were transferred to liquid media and cells in exponential phase were used for protoplast isolation. In both of Dejima and Superior, the yield of protoplasts was high in the enzyme solution of 2% Onozuka cellulase and 1% macerozyme. Also, viability of isolated protoplasts was very good. Thus, it seems that these protoplasts would be applicable to various aims of research.

INTRODUCTION

Protoplasts isolated from various plants could be regenerated into the whole plants. Also, they could form the interspecific hybrids by fusion (Sink, 1984) and could uptake the foreign materials such as cell organelles or DNA (Krens *et al.*, 1982).

Because of these great advantages, diverse investigation using protopasts has been progressed for the improvement of food crops (Dale, 1983) in which the potato ranks fourth (Wade, 1975). Regeneration abilities of protoplasts belonging to the family Solanaceae were higher than those of other families (Ahuia, 1982). In 1977, Shepard and Totten succeeded in regeneration of potato mesophyll protoplasts into whole plants. Since then, there were several reports on the regenerated plants from isolated potato mesophyll protoplasts (Binding *et al.*, 1978; Thomas, 1981; Kikuta *et al.*, 1983). There were many examples of the regenerated plantlets from potato tuber tissue cultures (Lam, 1975, 1977a; Skirvin *et al.*, 1975; Jarret *et al.*, 1980; Kikuta and Okazawa, 1982). However, there was no report that protoplasts isolated from potato tuber tissue were regenerated into whole plants.

Crown gall tumors were induced in potato tuber tissues by infection with the soil bacterium *Agrobacterium tumefaciens* (Cha *et al.*, 1983). The tumor induction involves transfer of a portion of the Ti plasmid DNA from the bacterium to the plant cells (Chilton *et al.*, 1980; Wullems *et al.*, 1984). Because *in vitro* transformation of tobacco protoplasts with Ti plasmid DNA was successful (Krens *et al.*, 1982), it seems likely that transformation of potato protoplasts with Ti plasmid DNA could be also possible.

In our present work, it was attempted to isolate efficiently the protoplasts from potato tuber tissue to use them as fusion partner or host cell for foreign gene transfer.

MATERIALS AND METHODS

Plant materials. The potato (*Solanum tuberosum* L.) tubers used were cultivars, Dejima, Irish Cobbler, Shimabara and Superior which were stored at 4° C until use after harvest. They were kindly provided by Horticultural Experiment Station, O.R.D., Suweon, Korea.

Callus induction. Tubers weighing between 200 g and 300 g were scrubbed, washed with water, surface-sterilized with 5.25% sodium hypochlorite solution for 15 minutes and rinsed in sterile water. The perimedullary tissue cylinders (6 mm or 10 mm, in diam.) were taken longitudinally from tuber with a cork borer and the terminal 5 mm sections were severed and discarded. Discs of the tissue (1 mm to 5 mm thick) were sliced and immediately washed several times in sterile water. After removal of excess water with sterile filter paper, each tuber disc was aseptically transferred to a 100 ml Erlenmeyer flask containing 20 ml of the basal medium of Lam (1975) in which $MnCl_2.4H_2O$ (19.2 mg/l) and $CoSO_4.7H_2O$ (0.03 mg/l) were substituted for $MnSO_4.4H_2O$ (22.3 mg/l) and $CoCl_2.6H_2O$ (0.025 mg/l), respectively, after Shepard and Totten(1977), and 2, 4-D (3 mg/l) was also added. The cultures were kept in darkness for 2 days, then transferred to plant growth chamber (Percival Mfg. Co., USA) with an intensity of 8,500 lux of fluorescent light, a photoperiod of 16 hours and a constant temperature of 27° C. The calli formed after 5~6 weeks of culture were subcultured on the same nutrient media.

Suspension culture. About 1.5 g of friable callus was inoculated into 50 ml of liquid medium of Lam (1977b) in 250 ml flask. The liquid suspension was agitated continuously at 78 rev/min on a reciprocal shaker under continuous light of 1,000 lux at 30°C. After 10 days of culture, the suspended cells were filtered through one layer of cheesecloth in order to collect the single cells. 5 ml of single cell-suspension was pipetted into the 100 ml flask containing 15 ml of fresh medium and its growth curve relating packed-cell volume (PCV) per unit volume to time was measured. A known volume of suspension was transferred to a 10 ml graduated conical centrifuge tube and centrifuged at 2,000 *g* for 5 minutes, and the volume of cells was expressed as PCV(ml) per culture.

Since only a few cells were divided actively in liquid medium of Lam, slightly modified liquid media were tested to obtain much more actively dividing cells for protoplast isolation

Table 1. Different compositions for suspension culture media

	Media (mg/l)					
	1	2	3	4	5	6
Inorganic salts						
major element	M.S§	M.S	M.S	M.S§	M.S§	M.S§
minor element	1/5 of M.S	M.S	M.S	1/5 of M.S	M.S	1/5 of M.S
Organic constituents						
organic addenda	N.N	N.N	N.N	N.N	N.N	N.N
Casein hydrolyzate	1000	1000	1000	1000	1000	1000
Sucrose	1%	2%	2%	1%	3%	2%
Glucose	1%	—	—	1%	—	—
Mannitol	4%	—	—	4%	—	—
Growth regulators						
BAP	0.5	—	—	—	—	—
Kinetin	—	0.8	—	—	0.25	—
GA_3	0.2	0.4	—	—	—	—
2,4-D	2.0	3.0	2.5	2.5	2.0	2.5
IAA	—	0.4	—	—	—	—

M.S; Murashige and Skoog (1962) medium
M.S§; M.S without NH_4NO_3
N.N; Nitsch and Nitsch (1969) medium

(Table 1). In this case, 1 g of friable callus was transferred to 250 ml flask containing 50 ml of experimental medium. It was then cultured for 9 days under same conditions as above and observed.

Isolation of protoplasts. Each culture of suspended cells was subcultured twice before they were used as source of protoplasts, each subculture passage lasting 7 days. Cell samples were obtained by centrifuging at 100 *g* for 5 minutes. 100 mg of collected cells was then resuspended in 5 ml of enzyme solution contained in 60 mm Petri dish and agitated slightly on a shaker for 5 hours at 27° C. Enzyme solution consisted of 0.5 M mannitol, Onozuka cellulase R-10 (Kinki Yakult Co., Nishinomiya, Japan), macerozyme R-10 (Kinki Yakult Co.) and Murashige and Skoog nutrient salts (without NH_4NO_3). pH was adjusted to 5.7 with KOH. After incubation, the undigested cell aggregates were first filtered through 103 μm pore size stainless steel mesh and then through 87 μm mesh. The filtrate was transferred to a conical centrifuge tube and centrifuged at 75 *g* for 5 minutes. Pellet portion of protoplasts was then resuspended in 0.5 M mannitol solution containing nutrient salts. The number of protoplasts was determined with hemacytometer. Viability was assessed by examining the ability to exclude Evans Blue (Kanai and Edwards, 1973; Redenbaugh *et al.*, 1981; Saga, 1984). 0.4% Evans Blue solution including 0.5 M mannitol was mixed with

an equal volume of protoplast solution and examined under light microscope.

RESULTS AND DISCUSSION

Abilities of callus formation of different potato cultivars. There were some differences in callus-forming stage and morphological pattern among cultivars. Callus induction of Dejima was more rapid than that of other cultivars. Calli formation of all cultivars was initiated within 7 days of culture. Calli were generally induced from edge of the tuber discs, except in the case of Shimabara, for which calli were formed at upper surface evenly and homogeneously. Ability of callus formation was dependent on thickness but not diameter of tuber discs. It was observed that callus-forming activities were efficient in order of 2 mm, 1 mm, 3 mm, 4 mm and 5 mm thickness at both of 6 mm and 10 mm diameter of tuber discs. Fresh weight of calli relating time in different cultivars are shown in Fig. 1. The initial disc weight of 10×2 mm size was increased about 4.6 times in Irish Cobbler, 4.2 times in Shimabara, 6.5 times in Superior and 7.8 times in Dejima, respectively, after 35 days of culture. Since abilities of callus formation in Dejima and Superior were better than those of Simabara and Irish Cobbler, the former was used as sources for protoplast isolation. Usually, calli grew actively after induction of them and became friable after subculture. It was also observed that, in Dejima, subcultured callus became friable rapidly as compared with other cultivars.

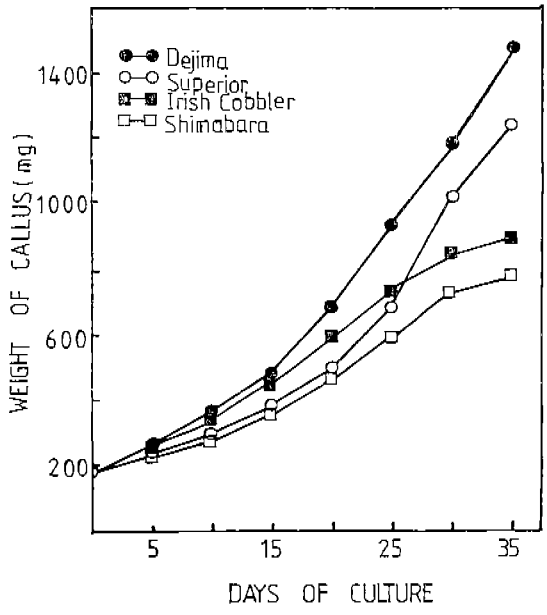

Fig. 1. Growth responses of the four different kinds of potato tuber callus.

In order to confirm the regenerative capacities of all the tuber discs, they were transferred to Lam (1977a) medium. As a result, it was observed that only Superior and Shimabara were regenerated into plantlets (Fig. 2). It appeared that another controlled media or growth conditions may be required for Djima and Irish Cobbler.

Cell suspension culture. Growth patterns of Dejima and Superior cells in suspension culture are shown in Fig. 3. Single cells of both of them at initial inoculation showed diversity in shape and size ranging from 150×70 μm to 200×100 μm in small cells and 400×70 μm to 650×100 μm in large cells. Cell aggregates were observed after 8 days of culture in both cultivars, and large

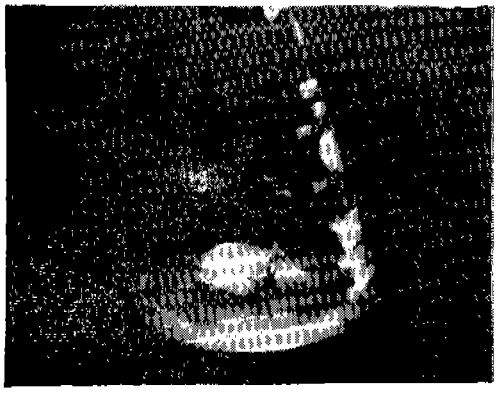

Fig. 2. Plantlet regenerated from potato tuber (cv. Superior) callus.

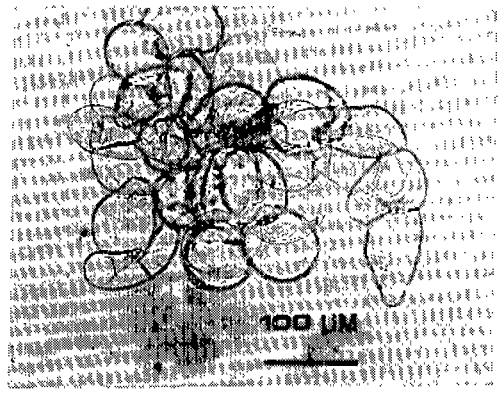

Fig. 4. Actively dividing cells in modified liquid medium after 9-day-old cell culture.

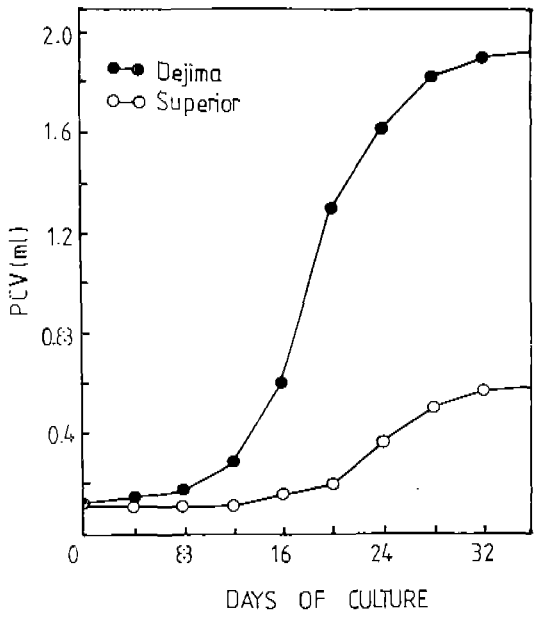

Fig. 3. Growth curve of the cell suspension grown under batch conditions.

cell clumps (1~3 mm, in diameter) were formed after 28 days of culture.

It was reported that rapidly dividing cell cultures are the most suitable source for protoplast isolation (Evans and Cocking, 1977). Thus, various combinations of medium components were tested (Table 1). Consequently, full strength of Murashige and Skoog minor inorganic salts, 3% sucrose as carbon source and 2.0 mg/l 2,4-D and 0.25 mg/l kinetin as growth regulators which were from Uchimiya and Murashige (1974) were substituted for original Lam's liquid medium. Fig. 4 shows that the cells were actively dividing in modified liquid medium after 9 days of culture. Therefore, this medium was thought to be optimal for protoplast isolation.

Isolation of protoplasts from suspension cultured cells. Several combinations of Onozuka cellulase and macerozyme were tested to determine the optimal concentration for protoplast isolation (Table 2). In both of Dejima and Superior, the yield of protoplasts was the highest when cells were incubated in the enzyme solution of 2% Onozuka cellulase and 1% macerozyme for 5 hours. Using a Nikon inverted microscope, the formation of protoplasts was observed at 1 hour interval through 7 hours. Protoplasts were liberated after

Table 2. Effect of enzyme concentration containing 0.5 M mannitol and M.S salts (without NH_4NO_3) on protoplast isolation. Incubations were performed at 27°C for 5 hours

Conc. of enzymes (%)		Amount of protoplasts isolated from 100 mg of suspension cell	
Onozuka cellulase	Macerozyme R-10	cv. Dejima	cv. Superior
1.0	0.2	6.5×10^4	7.0×10^4
2.0	0.5	9.0×10^4	1.1×10^5
2.0	1.0	1.0×10^5	1.4×10^5
3.0	1.0	1.0×10^5	1.2×10^5

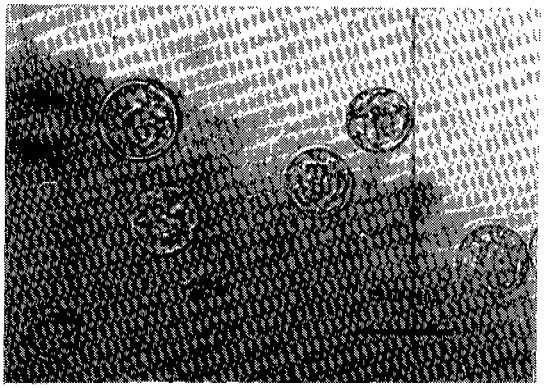

Fig. 5. Protoplasts isolated from cultured cells which were incubated in the enzyme mixture of 2% Onozuka cellulase, 1% macerozyme R-10, 0.5M mannitol and M.S salts (without NH_4NO_3) for 5 hours.

2 hours of incubation and maximally after 5 hours through 7 hours. The enzymes used for cell wall degradation are often contaminated with toxic substances such as various ribonucleases, proteolytic enzymes, lipases, peroxidases and nonenzymatic materials, and harmful effects on the protoplasts other than purely the removal of the cell wall may result (Cocking, 1972; Evans and Cocking, 1977). Ruesink (1980) also reported that the digestion time should be as short as possible when the tissues were incubated in enzyme solution for releasing protoplasts. Therefore, 5 hours incubation in enzyme solution was thought to be optimal for protoplast isolation. In the work reported by Tseng *et al.* (1975), the number of protoplasts from potato tuber callus tissue (1 g) which was incubated in an enzyme mixture (6 ml) containing 5% Onozuka cellulase, 2% macerozyme and 0.01% dextran sulfate in 0.6 M sucrose (pH 5.4) at 37° C for 5 hours was 5.0×10^4 per ml. In the present study, only 2% Onozuka cellulase and 1% macerozyme were sufficient to isolate more protoplasts when used in conjunction with 0.5 M mannitol and M.S. salts (without NH_4NO_3) at 27° C.

The protoplasts isolated from suspension cultured cells are shown as Fig. 5. The size of protoplasts was variable from 20 μm to 60 μm. Of them, 30 μm to 50 μm held above 60%. The number of protoplasts isolated from cell suspension was approximately 6~9 times as much as that of friable callus under same conditions.

Comparing the PCV of cell suspension with PCV of protoplasts isolated from it, the yield of protoplasts was 30~50%. In this study, it seems that the yield of protoplasts from cultured cells of potato tuber tissue is higher as compared with another report (Uchimiya

and Murashige, 1974) in which the yield of protoplasts from tobacco cells in suspension culture was about 30 %, being higher than the yields previously reported (Eriksson and Jonasson, 1969; Grambow *et al.*, 1972; Kameya and Uchimiya, 1972). Also, exclusion staining with Evans Blue showed that more than 90 % of the protoplasts was viable. Thus, it is thought that the protoplasts isolated from cultured cells of potato tuber tissue would be applicable to various aims of research. Studies on the regenerative capacities of protoplasts are in progress.

摘 要

감자(*Solanum tuberosum* L.) 괴경의 배양세포로부터 원형질체를 분리하였다. 감자 괴경을 Lam 배지에 배양한 결과, Dejima와 Superior 품종이 Shimabara와 Irish Cobbler 품종에 비해 callus의 형성능이 좋았으므로 전자를 원형질체 분리 재료로 선택하고 이들의 friable callus를 액체배지에 현탁배양 하면서 exponential phase의 세포로부터 원형질체를 분리하였다. Dejima와 Superior 두 품종 모두 2 % Onozuka cellulase와 1 % macerozyme의 효소용액에서 원형질체의 수율이 높았다. 또한 분리한 원형질체의 생존력도 매우 좋았다. 따라서 이들 원형질체는 여러가지 연구 목적에 이용 가능하다고 생각된다.

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