PROTOPLAST CULTURE OF POTATO: AN IMPROVED PROCEDURE FOR ISOLATING VIABLE PROTOPLASTS

Yoshio KIKUTA, Kaien FUJINO, Wataru SAITO, Kiyoshi MASUDA and Yozo OKAZAWA

(Department of Botany and Crop Physiology Faculty of Agriculture, Hokkaido University Sapporo 060, Japan)

Received January 10, 1986

Introduction

Since progress has been made in methods of isolation and manipulation of potato protoplasts, in suitable culture medium and environmental conditions the isolated protoplasts regenerate cellwalls, undergo cell divisions and may grow into complete plants (BINDING *et al.* 1978, KIKUTA *et al.* 1984, SHEPARD & TOTTEN 1977, THOMAS 1981). The application of cellwall degradation enzymes for the isolation of viable protoplasts made it possible to obtain a large quantity for experimental purposes (NAGATA & TAKEBE 1970). The complete digestion of the rigid cellwall and the complete exposure of the plasm membrane makes protoplasts a particularly useful system not only for producing somatic hybrids and manipulating the regeneration of plantlets but also for investigating the uptake of macromolecules and transport phenomena.

Protoplasts are usually produced by treating mesophyll tissue with a mixture of cellwall degrading enzymes in solutions which contain osmotic stabilizers to sustain the structure and function of the protoplasts. The most important factors for the liberation of potato protoplasts from leaves are the physiological state of material leaves, the kinds of degrading enzymes, the composition of the reaction solution and the concentration and type of osmotic stabilizers. In an attempt to achieve a highly reproducible isolation procedure for potato protoplasts we have investigated methods for protoplast isolation and purification. In this paper we report on an improved procedure for potato protoplast isolation using commercially available enzyme preparations and the application of a three-part Percoll/mannitol discontinuous gradient separation system which results in highly purified protoplast preparation for biochemical and physiological studies on plant development.

Materials and Methods

Plant Materials

A potato cultivar, *Solanum tuberosum* L. cv. May Queen, was used in the present study. Seed potatoes were purchased from a local market (Sapporo Kono-En, Sapporo, Japan), and grown in 10 cm pots with moistened vermiculite under 4,000 lx, 12 hr/day at 22°C. Expanded leaves were surface sterilized and mid-ribs were removed. They were cut into 1.0 mm strips and preincubated in 0.44 M mannitol solution for 2 hr in order to induce plasmolysis in mesophyll cells. Then they were ready for enzyme treatment.

Enzyme Purification

Cell-wall degradation enzyme preparations used were purchased from commercial sources. Cellulase ONOZUKA R10, Macerozyme R10 (Yakult, Nishinomiya Japan), Driselase (Kyowa Hakko, Tokyo Japan), Meicelase P-1 (Meiji Seika, Tokyo Japan), Pectolyase Y-23 (Seishin Seiyaku, Nagareyama Japan), and pectinase TCI (Tokyo Kasei, Tokyo Japan) were subjected to study for isolating protoplasts from potato leaves. These enzyme preparations were purified by chromatography on Sephadex G50 (Pharmacia Fine Chemicals, Uppsala Sweden), or used as they were.

Standard Incubation Medium and Procedure

For standard incubation conditions, the strips of potato leaves were immersed in 90 mm diameter plastic Petri dishes containing 8 ml of enzyme preparations, 0.44 M mannitol and 5 mM MES buffer, pH 5.4. The dishes were occasionally kept under gentle agitation at 25°C for more than 3 hr. For long-term incubations generally overnight, the incubation medium was replaced by new medium 5 hr after started, and was placed in an incubator at 20°C.

Purification of Potato Protoplasts

The incubation mixtures were filtered through a 44 μm mesh stainless steel sieve and collected by centrifugation at 95 xg for 3 min. The protoplasts thus obtained were resuspended in 2 ml of 0.44 M mannitol and 5 mM MES buffer, pH 6.0. This formed the top layer of a four-part discontinuous Percoll/0.44 M mannitol density gradient centrifugation system, which consisted of 2 ml each of 0%, 6%, 12%, 25% and 50% Percoll (Pharmacia Fine Chemicals, Uppsala Sweden) in 0.44 M mannitol with 5 mM MES buffer, pH 6.0. The system was centrifuged at 400 xg for 5 min to separate cell types and cell debris.

Measurement of Protoplast Populations

Protoplast densities and distributions were estimated with a 20 μl droplet counting method, using the mean of 6 determinations of the protoplasts present in 20 μl aliquots for each reported value. The counted number multiplied by 50 gives the protoplast density in ml.

Assessment of Protoplast Viability

Viability was assessed by the ability to accumulate (a) the vital dye stain neutral red (0.4% w/v) (b) fluorescein from a 0.01% v/v solution of fluorescein diacetate or to exclude the mortal stain Evans Blue (0.0025% w/v). In all cases stains were dissolved in resuspension mediu,. Cells were stained for 5 min before counting more than 10 random fields of view, each containing 50-80 protoplasts. Fragments of undigested cellwalls were visualised by staining protoplasts for β-linked polysaccharide with the fluorescent brighter Calcofluor White I (Dojin, Kumamoto Japan) and observing with a Nikon photomicroscope fitted with an epi-fluorescence attachment (LARKIN 1976, NAGATA & TAKEBE 1970).

Protoplast Regeneration

Protoplasts were cultured at 10^4/ml cell density in 6 cm plastic dishes containing 4 ml of protoplast culture medium. Cultures were incubated at 20°C in darkness and observed divided cells on day 7 and cell colonies on day 14. The composition of potato protoplast culture medium was as follows : (Mineral salts in mg/l) ; KNO_3 950.0, $CaCl_2 \cdot 2H_2O$ 1,220.0, $MgSO_4 \cdot 7H_2O$ 185.0, KH_2PO_4 85.0, FeEDTA 18.5, $MnSO_4 \cdot 4H_2O$ 10.0, $ZnSO_4 \cdot 7H_2O$ 4.6, H_2BO_3 3.1, KI 0.4, $NaMoO_4$ 0.1, $CoCl_2$ 0.02 and (Organic addenda in mg/l) ; thiamine 0.2, glycine 1.0, nicotinic acid 2.5, pyridoxine 0.25, folic acid 0.25, biotin 0.01, myo-inositol 50.0, casein hydrolysate 100.0, sucrose 1,000.0, NAA 1.5, zeatin 0.5, mannitol 63,000.0, and MES 1,000.0, pH 5.8. Cultures which formed cell colonies were diluted subsequently with fresh culture medium containing 0.22 M mannitol at 14 days intervals. Several colonies were transferred after 6 weeks in liquid culture to an agar medium sequence for callus proliferation and plantlet regeneration (KIKUTA & OKAZAWA 1984).

Results and Discussion

Cellulase and Pectinase Activities of Commercial Enzyme Preparations

Commercial enzyme preparations were purified by chromatography on Sephadex G50. 170 mg of enzyme preparation in 10 ml of 10 mM citrate buffer, pH 5.0 was fractionated on a column 25 × 450 mm, equilibrated with the same buffer and collected 3-ml fractions. Figure 1 shows the elution

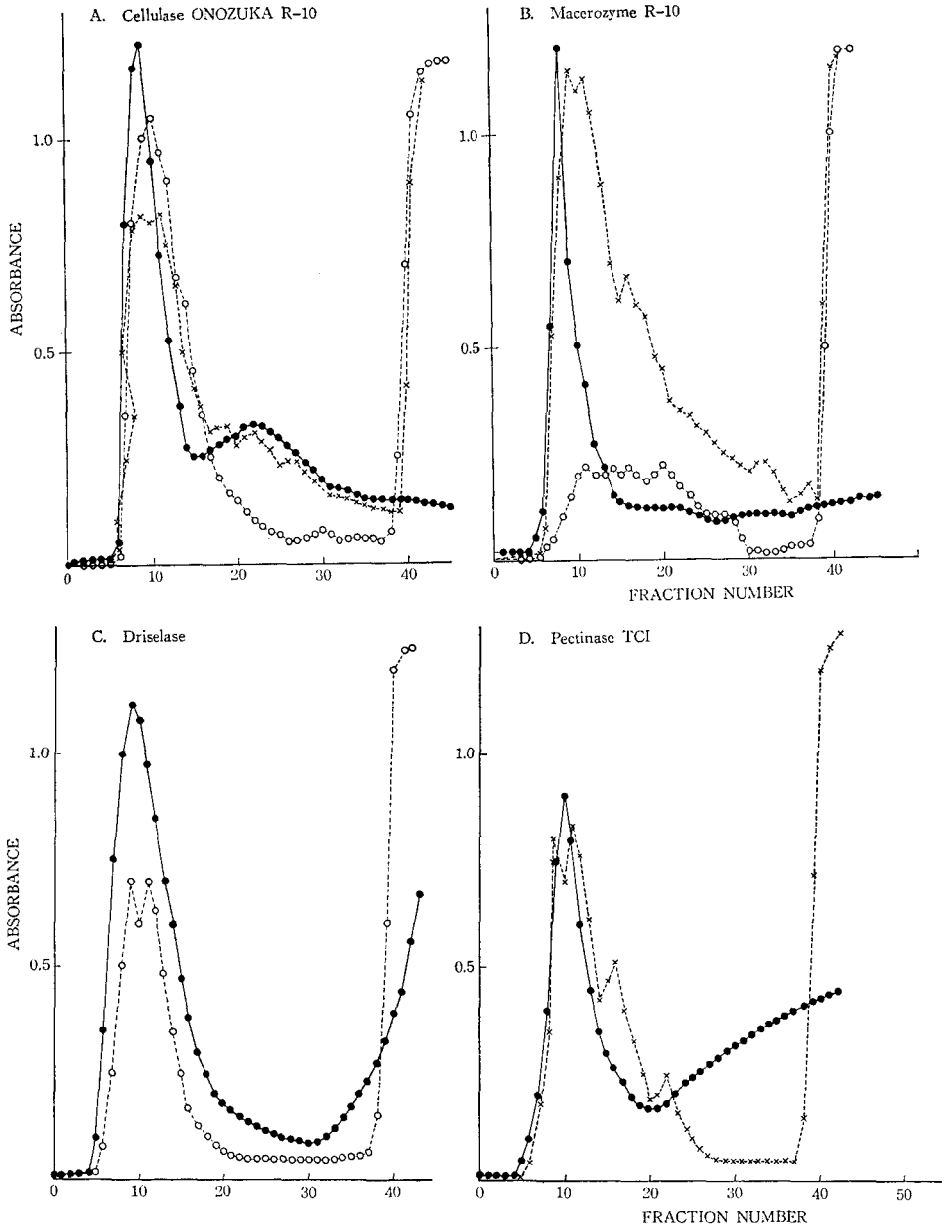


Fig. 1. Elution profiles of commercially available ennyme preparations on a Sephadex G50 column. The column was equilibrated with 10 mM citrate buffer, pH 5.0, and after loading the protein (●——●) was eluted with the same buffer and 3 ml fractions were collected. Cellulase (○·····○) and pectinase (×---×—) activities were assaysed and protted. (A) Cellulase Onozuka R-10, (B) Macerozyme R-10, (C) Driselase, and (D) Pectinase TCI.

patterns of 4 typical commercial enzyme preparations in which cellulase and pectinase activities are also protted in the figure. The first peak contained most of the protein as the effective ingradients for cellulase and pectinase activities. Cellulase activity was determined by an increase of reducing sugar units from a strip of Toyo filter paper No. 51, and pectinase activity by an increase of reducing sugar units from citrus pectin preparation washed by 80% ethanol and ice cold water. Table 1 summerised both activities and concentrations of the commercial enzyme preparations. Meicelase P-1, and Pectolyase Y-23 were not studied on their ingradients because they were expensive.

Applicability of Commercial Enzymes for Protoplast Isolation

As a matter of facts, only Onozuka R-10 cellulase contained all the activity required for potato leaf protoplast liberation. The activity of this enzyme preparation to liberate protoplasts without addition of other enzyme preparations is due to the presence of a sufficiently high concentration of pectinase in this preparation (Fig. 1. A and Table 1). Although the commercially available enzyme preparations are known to contain contaminants which are held to be deleterious to the protoplasts, these preparations are also known to have cofactors which stimulate cellwall degradation activity in a certain extent. Furthermore, the cellulase and pectinase activities were found in enzyme preparations as the indices for activity of the preparations but were not found to indicate a strict index for protoplast liberation. We decided to use these enzyme preparations, as they were in combination, in order to obtain the maximum activity for protoplast isolation.

TABLE 1. Cellulase and pectinase activities in commercially available enzyme preparations

Commercial name	% of Protein (%)	Specific activity			
		Cellulase activity		Pectinase activity	
		(AU/mg prot.)	(AU/mg prep.)	(AU/mg prot.)	(AU/mg prep.)
Cellulase					
Onozuka R-10	28.1	4.92	0.46	3.43	0.32
Macerozyme R10	8.2	2.34	0.06	8.13	0.22
Driselase	13.0	3.13	0.14	nd	nd
Pectinase TCI	2.4	nd	nd	16.06	0.35

Meicelase P-1 and Pectolyase Y-23 were not studied.

AU: Arbitary units, Change of absorbance at 520 nm produced by Somogii reaction with reducing sugars which appeared during 4 hr incubation per mg protein or per mg preparation.

nd: Not determined.

In the initial work we found that usage of Onozuka R-10 cellulase preparation for protoplast production gave rise to variable results — particularly with respect to the extent of protoplast liberation. As a consequence of the results we tried to establish conditions for protoplast liberation using the mixture of Onozuka R-10 cellulase and macerozyme R-10 as a complementary enzyme source. Table 2 shows the most suitable combination of Onozuka R-10 cellulase and macerozyme R-10, 1.0 to 0.03 ratio, which is highly reproducible for the liberation of protoplasts.

TABLE 2. Enzyme solution tested for isolating protoplasts from May Queen potato leaves

I

Cellulase Onozuka R10 (%)	Protoplast density		Total purified
	in enzyme sol.	Banded 12/25	
3.0	11×10^3	28×10^4	23×10^5
1.0	17×10^3	43×10^4	36×10^5
0.3	11×10^3	28×10^4	23×10^5
0.1	10×10^3	25×10^4	21×10^5

0.1% Macerozyme R-10 was added tothe incubation mixture.

II

Macerozyme R10+1% Cellulase Onozuka R10 (%)	Protoplast density		Total Purified
	in enzyme sol.	Banded 12/25	
0.3	6.8×10^3	34×10^4	18×10^5
0.1	10.3×10^3	52×10^4	27×10^5
0.03	8.2×10^3	41×10^4	22×10^5
0.0	4.6×10^3	23×10^4	12×10^5

1.0% Cellulase Onozuka R-10 was added to the mixture.

This enzyme mixture is now applicable for isolating protoplasts from leaves of potatoes, tomatoes, petunia and tobacco plants. We also found that Macerozyme R-10 could be replaced by Pectolyase Y-23. Furthermore, the osmotic conditions and incubation duration were determined by the production of spherical protoplasts from plasmolysed mesophyll tissue (Table 3 and 4). Besides these data, the incubation temperature, pH, and reaction time, during which the intact plasma membranes were exposed to the degradative enzymes such as proteases, nucleases, and phospholipases as the impurities of commercial preparations, were really important to preserve the viability of isolated protoplasts.

TABLE 3. Osmotic condition of enzyme reaction mixture tested for isolating protoplasts from May Queen potato leaves

Osmotic concentration

Mannitol concen. (M)	Solute potential (MPa)	Protoplast density (Protoplast/g leaves)
0.3	−0.765	5.5×10^4
0.4	−1.024	18.5×10^4
0.5	−1.275	23.5×10^4
0.6	−1.530	26.9×10^4
0.7	−1.785	24.3×10^4

The sterilized strips of potato leaves were incubated in enzyme mixture containing 1.0% Cellulase Onozuka R-10, 0.1% Macerozyme R-10, various molar concentrations of mannitol and 5 mM MES (2-N-morpholino-ethanesulfonic acid) buffer, pH 5.4, with shaking 75 rpm at 25°C for 5 hr.

TABLE 4. Duration of cell-wall degration enzyme reaction tested for isolating protoplasts from May Queen potato leaves

Reaction Time (hr)	Protoplast production (protoplasts/g leaves)
0	0
0.5	1.0×10^4
1.0	6.0×10^4
2.0	23.5×10^4
3.0	30.0×10^4
4.0	28.6×10^4
5.0	21.5×10^4

The sterilized strips of potato leaves were incubated in enzyme mixture containing 11.0% Cellulase Onozuka R-10, 0.1% Macerozyme R-10, 0.55 M mannitol and 5 mM MES buffer, pH 5.4, wth shaking 75 rpm at 25°C for 5 hr.

Procedure for Protoplast Isolation

Our improved procedure for protoplast isolation is now described: expanded upper leaves, the plants were cultured under dim light of 3,000 lx 8 hr/day at 23°C, were harvested right before isolating protoplasts, sterilized with 70% ethanol for 30 sec and immersed in 10% Sodium hypochlorite solution (effective chlorine was 0.5%) with a drop of Tween #20 for 5 min. The leaves were washed with sterilized water for several times and brotted dry with sterile filter papers. Then mid-ribs were removed and epidermis

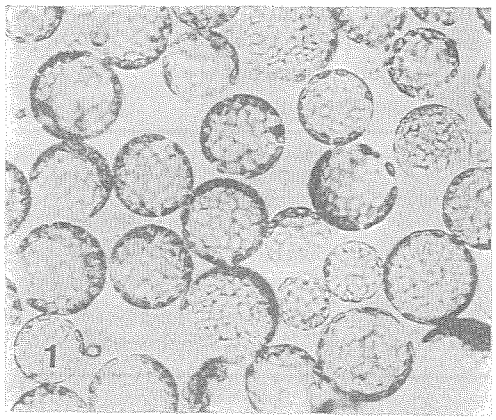
were cut into 1 mm wide strips which were then preincubated in 0.44 M mannitol containing 5 mM MES buffer, pH 6.0 for 2 hr. The plasmolyzed leaf strips were incubated with the enzyme mixture containing 1% Cellulase Onozuka R-10, 0.03% Macerozyme R-10, 0.44 M mannitol and 5 mM MES buffer, pH 5.4 with shaking 75 rpm at 25°C for 5 hr. When the system did not work well, the enzyme mixture was supplemented with 0.03% Pectolyase Y-23, 0.03% Driselase, 0.1% Pectinase TCI and 0.1% bovine serum albumin FrV and with modified White medium containing 0.5 mg/l zeatin and 1.5 mg/l NAA, and incubated at 20°C for overnight (total 16 hr). The enzyme mixture was passed through 0.45 μm Millipore filters (HAWPO25; Millipore Co. Bedford Mass USA) prior to employment for isolating protoplasts. Thus the liberated protoplasts were separated through a 44 μm mesh stainless steel sieve and collected by centrifugation at 100 xg for 5 min, and lacked any detectable cellwall when assayed using Calcofluor White I.

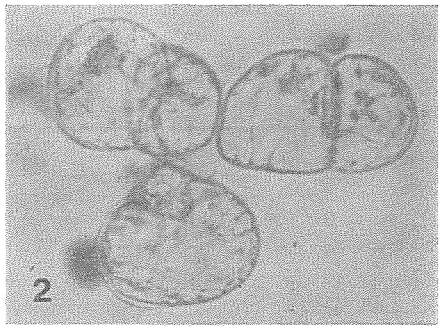
Protoplast Purification

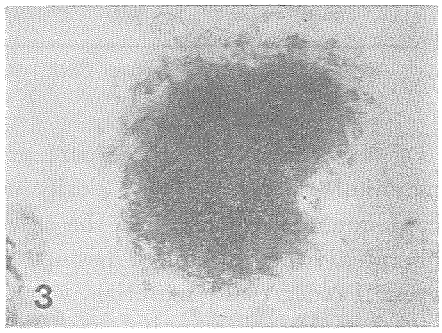
Protoplasts can be purified from other cellular debris by flotation on sucrose (SHEPARD & TOTTEN 1977). On several occasions the protoplasts did not float and separation of other components was negligible by centrifugation. A much more potentially useful technique for protoplast purification is based on the Percoll density gradient separation without much changes in solute potantials. In the first experiment, a four-part discontinuous Percoll gradient was used to separate protoplasts from mesophyll cells and epidermis cells from cell debris, i.e., mostly chloroplasts and nuclei from the broken protoplasts during enzyme treatment, at the end of a full incubation period. Table 5 shows the protoplast distribution on the Percoll/0.44 M mannitol density gradient separation at 400 xg for 5 min. Epidermal protoplasts were found to have the lowest density, usually appearing above the 12% Percoll layer, while mesophyll protoplasts were found to appear above the 25% Percoll layer.

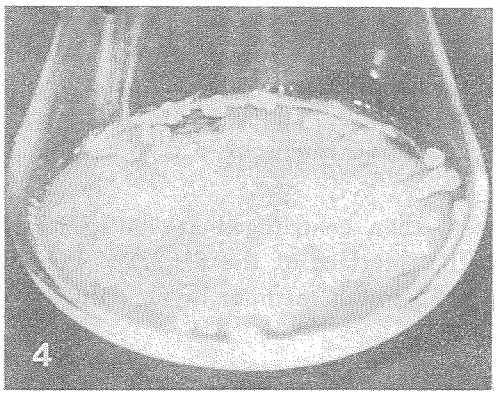
Fig. 2. Development of potato protoplast culture.

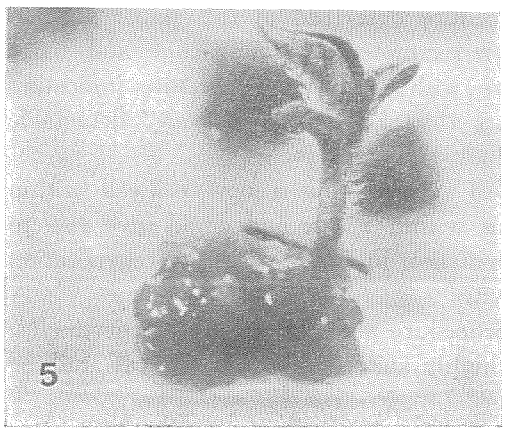
1. Over 90% potato, *Solanum tuberosum*L. cv May Queen, protoplasts were viable after 5 hr in 1% Onozuka R10 cellulase and 0.03% Macerozyme R10 0.44 M mannitol and 5 mM MES buffer, pH 5.4.
2. Protoplast cultured without Ca^{++}, zeatin and NAA died within four days when cell divisions were initiated.
3. Sucrose, glucose and NH_4NO_3 in ample amounts were not required for sustaining cell divisions and colony formation.
4. Cell colonies cultured with 1 mg/l zeatin and 0.3 mg/l IAA gave the highest frequency of shoot-bud formation.
5. Shoot-bud appeared in 2 months after transfer.
6. Regenerated plant gave small tubers.


1


2


3


4


5

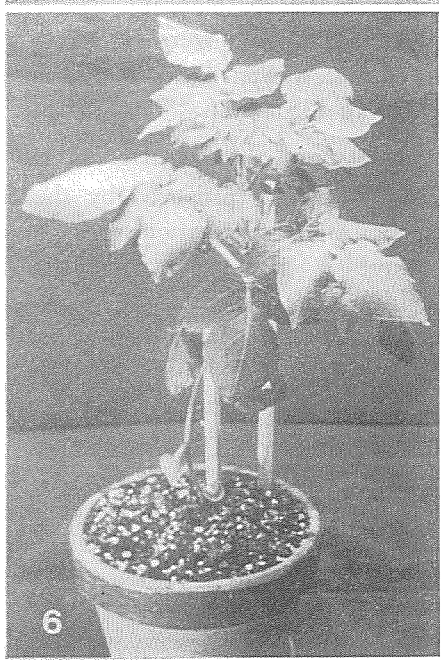

6

TABLE 5. Protoplast distribution on Percoll/mannitol discontinuous density gradient separation and properties of Percoll at varous concentrationsin 0.44 M mannitol at 20°C

% Percoll in 0.3 M mannitol (%)	Solute Potential (MPa)	Density (g/ml)	Mesophyll Protoplast	Epidermal Protoplast
0	−1.122	1.025		50×10^2
6	−1.125	1.033	50×10^2	20×10^2
12	−1.129	1.041	50×10^2	20×10^2
25	−1.135	1.058	30×10^2	
50	−1.145	1.090		

Values are calculated from GRT=2.55
G: Cryoscopic coefficient

As a routine, a three-part gradient of 0%/6%/25% Percoll in 0.44 M mannitol was employed to purify mesophyll protoplasts from any type of cell contamination present. In the case described above, almost all the protoplasts liberated from mesophyll tissue appeared at the 6%/25% Percoll interface, even if older plant materials were used.

The Percoll/mannitol gradients were then centrifuged at 400 xg for 5 min. Protoplasts were removed from the interface of the 6%/25% Percoll layer and washed twice in the appropriate growth medium by centrifugation at 95 xg for 2 min.

Viability and Regeneration of Isolated Protoplasts

Protoplasts prepared by the procedure described in this paper showed negligible contamination present when examined by microscope and more than 90% protoplasts stained positive with fluorescein diacetate indicating a high cell viability. The protoplasts thus isolated lacked any detectable cellwall when examined using Calcofluor White I.

These protoplasts were capable of regenerating a new cellwall within 24 hr and of initiating DNA synthesis within 72 hr, and could give rise to cell colonies followed by shoot-bud formation in Figure 2, plant regeneration from mesophyll protoplasts was shown.

It is concluded that use of this procedure allows the highly reproducible production of protoplasts which are highly suitable for biochemical studies.

Summary

A procedure is reported for isolating viable protoplasts from potato leaves. The method utilizes the mixture of Cellulase Onozuka R-10 and

Macerozyme R-10, containing 0.44 M mannitol and 5 mM MES buffer, pH 5.4 with shaking 75 rpm for 5 hr at 25°C. Intact protoplasts are separated from cellular debris by application of a three-part Percoll/mannitol discontinuous gradient system. The protoplasts isolated in this procedure lack any detectable cellwall and more than 90% viable when assayed using Calcofluor White I and fluorescein diacetate. It is concluded that such protoplasts are highly suitable for biochemical and physiological studies on plant development and transformation.

Acknowledgements

The work was in part supported by a Grant-in-Aid for General Scientific Research (B) 60480032 from the Ministry of Education, Science and Culture of Japan. K. FUJINO is a Research trainee from Sapporo Breweries Co. and W. SAITO with Kikkoman Co.

References

BINDING, H., NEHLS, R., SCHEIDER, O., SOPORY, S. K. and WENZEL, G.: Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum*, *Physiol. Plant.* **43**: 53-54. 1978

KIKUTA, Y. and OKAZAWA, Y.: Control of root and shoot-bud formation from potato tuber tissue cultured *in vitro*, *Physiol. Plaut.* **61**: 8-12. 1984

KIKUTA, Y., SAITO, W. and OKAZAWA, Y.: Protoplast culturs of potato: Changes in viability and initiation of cell division, *Plant Tissue Culture Lett.* **1**: 18-21. 1984

LARKIN, R. J.: Purification and viability determination of plant protoplasts, *Planta* **128**: 213-216. 1976

NAGATA, T. and TAKEBE, T.: Cellwall regeneration and cell division in isolated tobacco mesophyll protoplasts, *Planta* **92**: 301-308. 1970

SHEPARD, J. F. and TOTTEN, R. E.: Mesophyll cell protoplasts of potato. Isolation, proliferation, and plant regeneration. *Plant Physiol.* **60**: 313-316. 1977

THOMAS, E.: Plant regeneration from shoot culture derived protoplasts of tetraploid potato (*Solanum tuberosum* cv. Maris Bard). *Plant. Science Lett.* **23**: 81-88. 1981