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Protoplasts of *Marchantia polymorpha* Are Stabilized by Low Concentrations of Cellulase in the Medium During the Early Stage of Culture

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Protoplasts isolated from callus tissues of *Marchantia polymorpha* L. were cultured in medium with cell wall degradating enzymes containing cellulase activity. Survival of protoplasts decreased rapidly in medium without enzymes, whereas addition of low concentrations (0.01-0.1%) of enzymes prevented decrease in protoplast survival. Protoplasts precultured in medium with enzymes for 3 days showed a high rate of division when transferred to medium for division of protoplast-derived cells. Possible involvement of the regenerated cell wall in destabilization of cultured protoplasts was discussed.

Introduction

Plant protoplasts exhibit coagulation of cytoplasm, vacuolation and eventually bursting as symptoms of injury when cultured under sub-optimal conditions. However, the mechanism involved in this destabilization of cultured protoplasts is not clear. Therefore, in studies using plant protoplast cultures, great effort has been directed to determining optimal culture conditions by changing components of protoplast culture media such as plant growth regulators. Different approaches have been performed in order to stabilize cultured protoplasts and eventually enhance the division of protoplast-derived cells by several researchers[1-4]. We recently demonstrated that several cell wall staining dyes such as Calcofluor White[5] and inhibitors of cell wall biosynthesis such as coumarin[6] stabilize protoplasts of *Marchantia polymorpha*, and these substances rather enhance the rate of division of the protoplast-derived cells. In these studies, we suggested that the process of cell wall regeneration and/or regenerated cell wall *per se* is closely associated with the mechanism of destabilization of cultured protoplasts of *M. polymorpha*[5,6].

In the present report, we describe that *Marchantia* protoplasts cultured in the presence of cellulase at lower concentrations than those used in usual protoplast isolation maintained a high viability without suffering injury during the early stage of culture, suggesting that the regenerated cell wall *per se* plays an important role in destabilization of the protoplast during this stage.

Materials and Methods

Protoplasts were isolated from callus culture of *Marchantia polymorpha* L. as described previously[4,5]. Callus of *M. Polymorpha* were subcultured at monthly intervals on Murashige-Skoog's (MS) medium[7] containing 1 mg/*l* 2,4-D, 0.1% (w/v) yeast extract (Difco, USA), 30 g/*l* sucrose and 0.8% (w/v) agar. To isolate protoplasts, 2 g of callus tissue which had been cultured for 6-8 days after subculture were incubated with shaking in 20 m*l* of 0.7 M mannitol containing 2% (w/

v) Driselase (Kyowa Hakkou Kogyo, Japan), pH 5.5 at 30°C for 2 hrs. The isolated protoplasts were filtered through 62 μm nylon net and washed twice by centrifugation at 40×g for 3 min. with 0.7 M mannitol and then once with liquid protoplast medium (P-medium)[5], which was composed of MS medium containing 1 mg/l 2,4-D, 0.1% yeast extract, 0.23 M mannitol and 30 g/l sucrose. After washing, 2 ml of protoplast suspension in P-medium was spread on 8 ml of solid P-medium (1% agar) in a 60 mm glass petri dish at final density of 1-2×10^4 protoplast/ml medium. All the enzymes used were dissolved in P-medium to 1 or 5% (w/v) and sterilized through membrane filter (0.22 μm pore size) after adjustment to pH 5.7. Aliquots of enzyme solutions were added to protoplast suspensions on the solid P-medium to the final concentration. Activated charcoal (Wako Junyaku, Japan) was added to solid P-medium (1.2% agar) before autoclaving. Protoplasts were cultured at 24±1°C in the light at about 1.5 W/m^2. Survival of cultured protoplasts was determined after staining with FDA (fluorescein diacetate) (Sigma Chemicals Co., USA)[8]. Regenerated cell walls in the protoplasts were observed by staining with Calcofluor White ST (American Cyanamid Co., USA)[9].

Results

The survival rate of *Marchantia* protoplasts changed during culture depending on the presence of Driselase in the medium (**Fig. 1**). When protoplasts were cultured without Driselase, the survival rate of protoplasts decreased to about 50 or 20-30% after 3 or 6 days of culture, respectively. Addition of Driselase to protoplast culture medium prevented the decrease in protoplast survival (**Fig. 1**). Each concentration of Driselase examined was equally effective in the first day of culture, however, effects of differing concentrations of Driselase on protoplast survival became manifest after 3 days. At 0.01 or 0.05% of Driselase, a high rate of protoplast survival was maintained to 3 days of culture, however, a rapid decrease in survival was observed even at these concentrations after 6 days of culture.

As shown in **Fig. 2**, regeneration of cell walls as detected by staining with Calcofluor White ST[9] occurred in protoplasts cultured on P-medium without Driselase (control) (**Fig. 2-B, C**). Little or no fluorescence of stained cell wall could be observed in protoplasts cultured with either concentration of Driselase by 3 days of culture (**Fig. 2-D, E**). A faint fluorescence was detected in protoplasts cultured with 0.01% Driselase after 6 days of culture (**Fig. 2-F, G**), and protoplasts at this

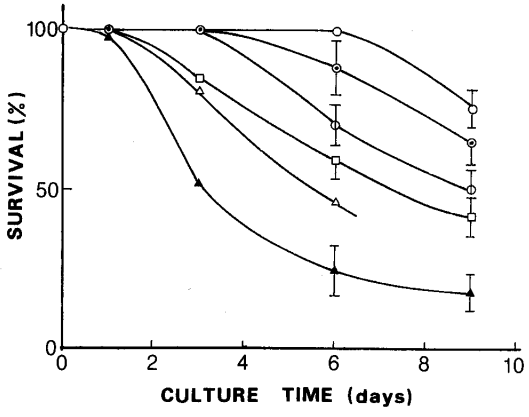

Fig. 1 Changes in protoplast survival during culture in the presence of different concentrations of Driselase in the medium.
Driselase concentrations are as follows: ▲—▲, 0%; ○—○, 0.01%; ⊙—⊙, 0.05%; ①—①, 0.1%; □—□, 0.5%; △—△, 1.0%. Bars indicate S.E. (n=3 or 4).

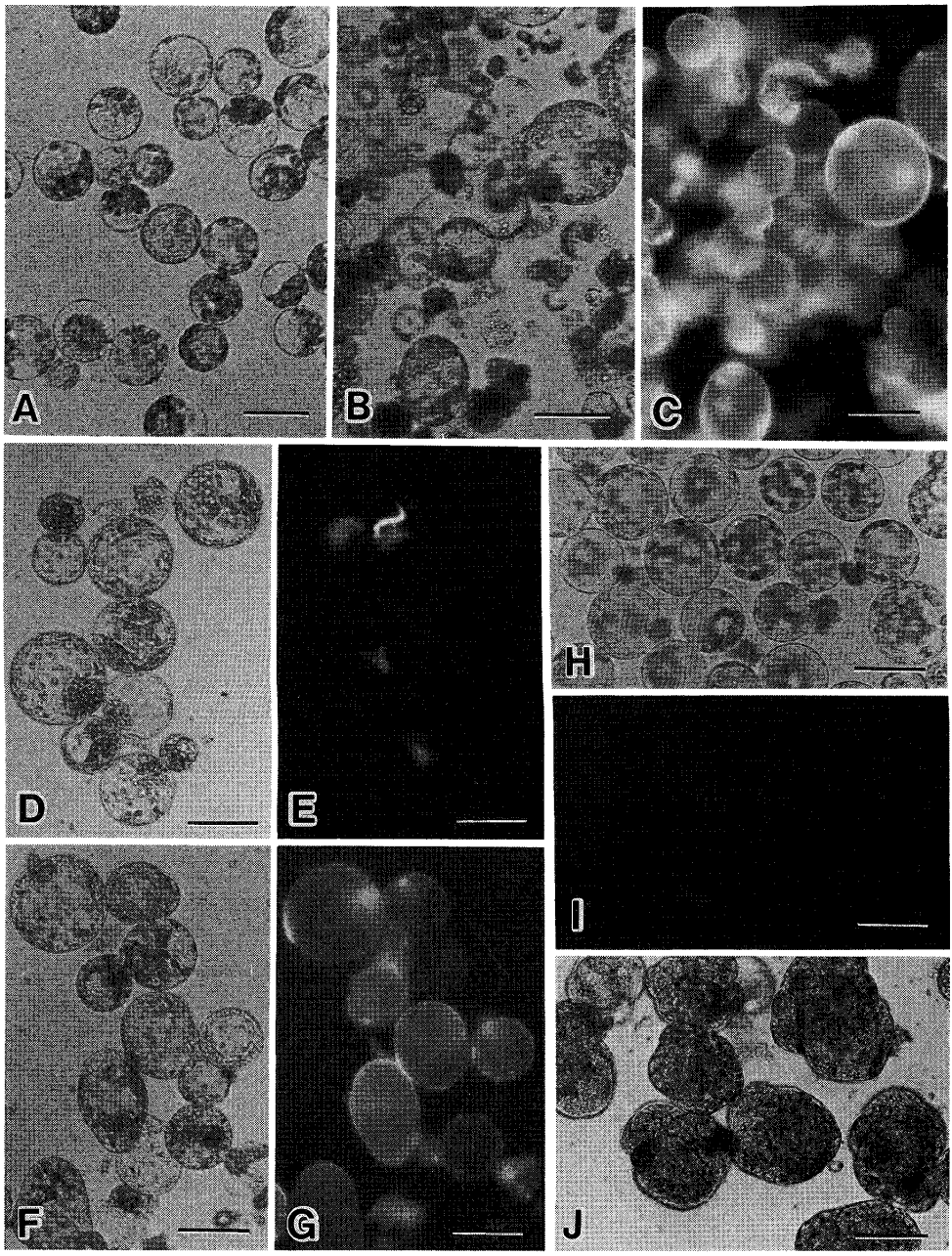

Fig. 2 Observations of *Marchantia* protoplasts under a light microscope (A, B, D, F, H and J) and fluorescence microscope (C, E, G and I).
A, freshly isolated protoplasts; B and C, protoplasts cultured for 3 days in medium without enzyme (control); D and E, protoplasts cultured with 0.01% Driselase for 3 days; F and G, Protoplasts cultured with 0.01% Driselase for 6 days; H and I, protoplasts cultured with 0.5% Driselase for 6 days; J, cell clusters formed after 16 days of protoplast culture in medium with 1% activated charcoal, following 3 days of culture with 0.05% Driselase. Protoplasts (C, E and G) were observed after staining with 0.1% Calcofluor White ST in 0.4 M mannitol. Bars indicate 50 μm.

culture stage showed budding or an ellipsoidal from (**Fig. 2-F, G**), indicating that the deposition of cell wall materials on the protoplast occurs in protoplasts cultured even with 0.01% Driselase after 6 days of culture.

Table 1. Effect of cell wall degradating enzymes on the survival of *Marchantia* protoplasts during culture.

Enzymes	Concentration (%)	Survival (%) *1	Cell wall *2
Control (no enzyme)	–	39.1±3.1	+
Driselase	0.05	94.7±9.8	–
	0.01	99.4±3.8	–
Cellulase Onozuka R-10	0.05	86.4±0.7	–
	0.01	98.9±1.0	–
Cellulase (purified)	0.05	99.2±3.1	–
	0.01	97.7±1.6	–
Macerozyme R-10	0.05	41.4±2.5	+
	0.01	24.1±1.6	+
Hemicellulase	0.05	25.8±2.5	+
	0.01	26.3±1.9	+
Novozym 234	0.05	51.0±3.0	+
	0.01	46.5±2.4	+

Protoplasts were cultured in medium with designated concentration of enzymes for 4 days.
*1 Average±S. E. (n=3 or 4). *2 The presence of cell wall (+) was determined by the staining method using Calcofluor White ST[9].

The effects of several other enzymes on protoplast survival were examined. As shown in **Table 1**, Cellulase Onozuka R-10 (Yakult Honsha, Co., Ltd, Japan) and purified cellulase (55.3 units/mg DW, Worthington Biochemical Co., USA), which are both from *Trichoderma viride*, were equally effective as Driselase during 4 days of culture. However, little or no effect on protoplast survival was observed with Macerozyme R-10 (Yakult Honsha Co., Ltd, Japan), Hemicellulase (Sigma, USA) or Novozym 234 (Novo Nordisk A/S, Denmark). On the protoplasts cultured with Cellulase Onozuka R-10 little or no cell wall was observed, however, a few spots of fluorescence of cell wall stained with Calcofluor were observed on the surface of protoplast cultured with the purified cellulase. In protoplasts cultured with the latter three enzymes, the regeneration of cell wall was observed to the same extent as protoplasts cultured without these enzymes (control protoplast).

Marchantia protoplasts used in the present study require activated charcoal in the medium for their division[4]. The absorption property of activated charcoal is well known, and it may absorb certain inhibitory substances of cell division[4,5]. However, the entity of the inhibitor (s) still remains unclear. In the present study, the rate of cell division of protoplasts which were cultured in the presence of enzymes was examined by replacing the medium with an enzyme with one containing activated charcoal. As shown in **Fig. 3**, the rate of cell division was very low (about 10%) in protoplasts cultured on P-medium without activated charcoal or enzymes. Only 15-20% cell division was observed in protoplasts which were cultured on P-medium for 3 days and then transferred onto medium with activated charcoal (**Fig. 3**). Approximately 50% cell division rate, which was comparable to that of protoplasts cultured with activated charcoal from the beginning of culture, was obtained in protoplasts which were cultured with Driselase or purified cellulase and then transferred to medium with activated charcoal (**Fig. 3**). On the other hand, very low cell division rates (10% or less) were observed in protoplasts cultured with Macerozyme R-10 or Hemicellulase even after transfer to medium with activated charcoal (**Fig. 3**).

Discussion

Our previous study[6] showed that coumarin and DCB (2, 6-dichlorobenzonitrile), which are well known as inhibitors of cellulose biosynthesis, inhibited the regeneration of cell walls of *Marchantia*

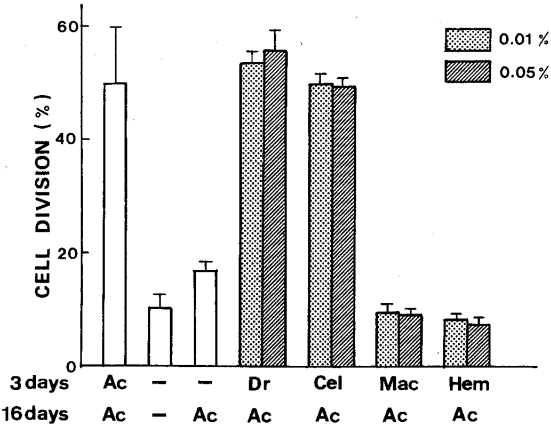

Fig. 3 Rates of cell division in protoplast cultures after replacing the medium with or without enzymes with medium containing activated charcoal.
Protoplasts cultured with or without enzymes for 3 days were washed with fresh medium and then transferred to medium with 1% activated charcoal and cultured for 16 days. Dr, Driselase; Cel, Cellulase (purified); Mac, Macerozyme; Hem, Hemicellulase; Ac, Activated charcoal; –, No additives. Final concentrations of enzymes were 0.01% (▒) and 0.05% (▓). Bars indicate S.E. (n=3 or 4).

protoplast when added to culture medium and simultaneously stabilized protoplasts during culture. The effects of these inhibitors were only observed during the early stage of protoplast culture[6]. On the basis of these results we deduced that regenerated cell walls *per se* and/or the process of cell wall regeneration play an important role in the destabilization of cultured protoplasts. However, it is not clear whether the destabilization of protoplasts is caused by the regenerated cell wall *per se* since the mechanism (s) and action site (s) of these inhibitors in the cell is not known[10,11], although a receptor for DCB has recently been revealed[12,13].

In the present study, it was further found that purified cellulase and cell wall digesting enzymes which contain cellulase activity all have the effect of protoplast stabilization during the early stage of protoplast culture (**Fig. 1, Table 1**) and that regenerated cell walls in the protoplasts cultured with these enzymes were removed with effective concentrations of the enzymes (**Fig. 2**). Furthemore, the effect of the enzymes was completely lost when the enzymes were autoclaved and no effect on protoplast stabilization was observed when cellobiose, which is an enzymatic product of cellulase, was added to the medium of protoplast culture instead of cellulase (data not shown). Therefore, these data suggest that cellulase in the enzyme preparations have a crucial role and removal of the regenerated cell wall by this enzyme is indispensable for the stabilization of cultured *Marchantia* protoplasts.

In *Marchantia* protoplast culture, more than 90% of protoplasts regenerated new cell walls within 24 hours of culture[4,5]. After this period, the rate of protoplast survival decreased rapidly in medium without enzymes (**Fig. 1**). There are many reports indicating that cell walls formed during the early stage of protoplast culture contain different components from those observed in the normal cell wall[14-17] and have an anomalous ultrastructure[18,19]. Klein *et al.* reported that a variety of polysaccharide-containing polymers other than cellulose were synthesized during the early stage of soybean protoplast culture and substantial amounts of 1, 3-glucan was contained in this polymer[16]. Takeuchi and Komamine have also revealed that the regenerated cell wall in protoplasts of *Vinca rosea* showed some differences from the cell wall of the original culture[17]. The biosynthesis of some of these components such as β-1, 3-glucan is known to be induced when

plants are wounded[16]. It is also suggested that an aberrant morphology of cultured protoplasts such as budding may be due to the result of uncoordinated assembly of the cell wall[14]. Recently, important roles of ECM (extracellular matrix) including polysaccharides and proteins have been suggested in various cellular functions of plant cells such as cytoskeleton-ECM continuum[20,21]. Therefore, it is most probable that the cell wall regenerated during the early stage of *Marchantia* protoplast culture have some defect(s) in structure and this leads to the decrease in protoplast survival.

As described earlier, *Marchantia* protoplasts require activated charcoal in the medium for the division of protoplast-derived cells. On the medium with activated charcoal, *Marchantia* protoplasts showed little or no decrease in their survival and about half of their protoplast-derived cells underwent cell division[4,5] (**Fig. 3**), suggesting that cellular functions in the protoplasts and protoplast-derived cells proceed normally in the presence of activated charcoal in the medium. These results suggest that there is some substance(s) which is inhibitory to the regeneration of normal cell wall and can removed by activated charcoal is involved in *Marchantia* protoplast culture. Further biochemical and ultrastructural studies are needed to clarify the mechanism of the destabilization of cultured *Marchantia* protoplasts.

References

1) Hahne, G., F. Hoffmann, 1984. Proc. Natl. Acad. Sci. USA, **81**: 5449-5453.
2) Saleem, M., A. J. Cutler, 1987. J. Plant Physiol., **128**: 479-484.
3) Carlberg, I., K. Glimelius, T. Eriksson, 1983. Plant Cell Rep., **2**: 223-225.
4) Sugawara, Y., K. Mori, H. Matsushima, M. Takeuchi, 1983. Z. Pflanzenphysiol., **109**: 275-278.
5) Fukukawa. E., Y. Sugawara, J. Plant Physiol., submitted.
6) Fukukawa, E., Y. Sugawara, J. Plant Physiol., submitted.
7) Murashige, T., T. Skoog, 1962. Physiol. Plant., **15**: 473-497.
8) Widholm, J. M., 1972. Stain Technol., **15**: 87-92.
9) Nagata, T., I. Takebe, 1970. Planta, **92**: 301-308.
10) Delmer D. P., 1983. Adv. Carbohydr. Chem. Biochem., **41**: 105-153.
11) Tarchevsky, I. A., G. N. Marchenko, 1991. In "Cellulose: Biosynthesis and Structure", p. 322, Springer-Verlag, Berlin-Heidelberg.
12) Delmer D. P., 1987. Annu. Rev. Plant Physiol., **38**: 259-290.
13) Delmer, D. P., S. M. Reed, G. Cooper, 1987. Plant Physiol., **84**: 415-420.
14) Hanke, D. E., D. H. Northcote, 1974. J. Cell Sci., **14**: 29-50.
15) Asamizu, T., A. Nishi, 1980. Physiol. Plant., **84**: 415-420.
16) Klein, A. S., D. Montezinos, D. P. Delmer, 1981. Planta, **152**: 105-114.
17) Takeuchi, Y., A. Komamine, 1978. Planta, **140**: 227-232.
18) Horine, R. K., A. W. Ruesink, 1972. Plant Physiol., **50**: 438-445.
19) Willison, J. H., E. C. Cocking, 1972. Protoplasma, **75**: 397-403.
20) Simmonds, D. H., 1992. Physiol. Plant., **85**: 387-390.
21) Wyatt, S. E., N. C. Carpita, 1993. Trends Cell Biol., **3**: 413-417.

《和文要約》

培養初期のゼニゴケプロトプラストは培地に添加した低濃度のセルラーゼによって安定化される

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ゼニゴケ(*Marchantia polymorpha* L.)のカルスから単離したプロトプラストをセルラーゼ活性のある細胞壁分解酵素を含む培地で培養した．プロトプラストの生存率は，酵素を含まない培地では急速に低下したが，低濃度(0.01～0.05%)の酵素を含む培地では低下が抑えられた．酵素を含む培地で3日間培養したプロトプラストは，活性炭を含む培地に移して培養すると高い分裂率を示した．培養プロトプラストの不安定化における再生した細胞壁の関与について考察した．