Journ. Fac. Sci., Hokkaido Univ. Ser. V (Botany), 13 (1): 7-16, 1983.

Natural and artificial production of protoplasts from heterothallic and homothallic *Closterium*

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Protoplasts were produced from the vegetative cells of *Closterium peracerosum-strigosum-littorale* (heterothallic) and *C. acerosum* (homothallic) by the treatment with 4% cellulase Onozuka R-10 and 0.5% Macerozyme R-10. The yields of the protoplasts from KAS-4-30 strain (mating type-plus) were higher than those from KAS-4-29 (mating type-minus) of the former species. In *C. acerosum*, it was only 8%. The protoplast productions from the mating type-plus and -minus cells were inhibited with more than 0.55 and 0.7 M sorbitol, respectively. During the process, chloroplasts were swollen and turned into spiral form in the protoplasts. Such behavior of the chloroplasts was not observed during the natural mating process of the heterothallic and homothallic strains and in vegetative cells of the homothallic strain. Cell wall was regenerated in the protoplasts of homothallic strain within 5 hr after removal of the lytic enzymes, but not in the heterothallic strains so far.

We have studied on changes in physiological and biochemical properties during the mating process of *Closterium acerosum* (homothallic) and *Closterium peracerosum-strigosum-littorale* (heterothallic) (KATO *et al.* 1981; SASAKI *et al.* 1976; UENO and SASAKI 1978 a, b). The vegetative cells have been known to convert into the sexually differentiated cells under the controlled conditions (ICHIMURA 1971; KATO *et al.* 1981; UENO and SASAKI 1978 a, b).

The algae, therefore, are interesing materials for the studies on the mechanisms of sexual differentiation, mating type cell-cell interaction and fusion, and nuclear fusion in green algae, also for the studies on sexually and asexually fused protoplasts.

We attempted to prepare biologically active protoplasts from vegetatively growing and sexually differentiated cells from the heterothallic and homothallic strains of *Closterium*. Several authors reported the production of protoplasts from desmids, *Cosmarium* (CHARDARD 1972) and other green algae (ADAMICH and SWEENEY 1976; GABRIER 1970; KOMBRINK and WÖBER 1980; MARCHANT and POWKE 1977; MÜLLER *et al.* 1978).

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Abbrebiation: MES, 2(N-morpholino) ethanesulfonic acid.

In this paper, procedures for efficient production of protoplasts from the heterothallic and homothallic strains of *Closterium* are described, and physiological significance of the differences between sexually opposite cells in susceptibility to cell wall digesting enzymes is discussed.

Materials and Methods

Algal materials

Closterium acerosum (homothallic) and *Closterium peracerosum-strigosum littorale* (heterothallic), KAS-4-29 (mating type-minus, mt^-) and KAS-4-30 (mating type-plus, mt^+), were grown in the growth medium with gentle stirring and in the mating medium without shaking according to ICHIMURA (1971) in a regime of 16 hr light (25°C) and 8 hr dark (20°C). Light intensity of 3,000 lux for vegetative growth or 10,000 lux for mating was fulfilled with cool white fluorescent tubes (Hitachi FLR-80-W/A). Using heterothallic strains, almost the same number of the mt^-- and mt^+-cells were mixed in the mating medium. The cells in late logarithmic growth phase or in early stationary phase were collected by low speed centrifugation, washed, and suspended in deionized water.

Preparation of protoplasts from vegetative cells

Protoplasts were prepared by the method of ITO (1973) with a minor modification. Standard incubation medium consisted of 4% cellulase Onozuka R-10, 0.4% Macerozyme R-10, and 0.4 M sorbitol in 0.03 M phosphate buffer (pH 5.5). Cells, 1×10^4 or 2×10^4/m*l*, were incubated at 20°C or 24°C with gentle shaking (10–15 or 50 stroke/min) in the dark. The number of protoplasts in 0.1 m*l* was counted and yield of protoplasts was calculated as % of total cell. The incubation medium was sterilized by filtration with a Millipore filter.

Regeneration of cell wall

Protoplasts produced by the treatment for 5 hr were transferred into growth medium containing 0.5 M sorbitol and further incubated at 25°C.

Chemicals

Cellulase Onozuka R-10 and Macerozyme R-10 were purchased from Yakuruto Pharmaceutical Industry Co, LTD.

Results

Protoplasts from C. peracerosum-strigosum-littorale

Protoplasts were able to be produced from vegetatively growing cells

with a high frequency by the treatment with 4% cellulase Onozuka and 0.5% Macerozyme in 0.5 M sorbitol at 24°C for 6 to 10 hr (Table 1). Such a combination of the two enzymes was the most effective, and MES buffer was the best among the buffer systems tested (Table 2). The mt^+-cells were more susceptible to the enzymes than the mt^--cells: in the mt^+-cells, the yields of protoplasts by 6 hr- and 10 hr-treatments were about 50 and 80%, respectively, and in the mt^--cells, the yields were 30 and 40% by the respective treatments (Tables 3 and 4). Sorbitol used as an osmotic stabilizer inhibited the protoplast productions from the mt^+- and mt^--cells at the

TABLE 1. Effects of different concentrations of cellulase Onozuka R-10 and Macerozyme R-10 on protoplast formation from vegetatively growing mt^- cells of heterothallic *Closterium*

No.	Enzymes (%)		Yields of protoplasts (%)		
			Incubation time (hr)		
	Cellulase	Macerozyme	4	6	10
1[a]	4	0.5	3.8	18.8	28.6
	5	0.5	3.2	6.4	9.1
	6	0.5	0.6	0.8	1.5
2[b]	4	0.4	—	16.6	24.2
	4	0.5	—	27.6	36.8
	4	0.6	—	0.1	0.0

Cells, 2×10^4/m*l*, were incubated in 0.5 M sorbitol and 0.03 M sorbitol and 0.03 M MES buffer (pH 5.5) at 24°C. a, Cells were grown for 14 days; b, Cells were grown for 16 days.

TABLE 2. Effects of different buffers at pH 5.5 on protoplast formation from vegetatively growing mt^+ and mt^- cells from heterothallic *Closterium*

Cells	Buffer (M)	Yields of protoplasts (%)	
		Incubation time (hr)	
		6	10
mt^+	MES (0.03)	50	79
	Phosphate (0.03)	33	60
	Citrate (0.03)	25	27
mt^-	MES (0.03)	28	42
	Phosphate (0.03)	16	18

Cells, 2×10^4/m*l*, were incubated with 4% cellulase Onozuka R-10 and 0.5% Macerozyme R-10 in 0.5 M sorbitol at 24°C. Cells were grown for 14 (mt^+) or 16 (mt^-) days.

TABLE 3. Comparison of efficiency of protoplast formation between vegetatively growing mt+ and mt− cells of heterothallic *Closterium*

Cells		Yields of protoplasts (%)		
Strain	Growth stage (Days)	Incubation time (hr) 6	8	10
mt+	14	50	—	79
	16	37	—	51
mt−	14	19	29	—
	16	28	37	42

Cells, 2×10^4/m*l*, were incubated with 4% cellulase Onozuka R-10 and 0.5% Macerozyme R-10 in 0.5 M sorbitol and 0.03 M MES buffer (pH 5.5) at 24°C.

TABLE 4. Effects of different concentrations of sorbitol on protoplast formation from vegetatively growing mt+ and mt− cells of heterothallic *Closterium*

Cells	Sorbitol conc. (M)	Yields of protoplasts (%) Incubation time (hr) 4	6–7	10	22–23
mt+[a]	0.50	Trace	36.5	51.3	55.8
	0.60	"	47.0	43.9	23.2
	0.70	"	3.2	4.8	9.5
	0.80	"	1.4	0.8	0.6
mt−[b]	0.45	"	15.6	20.7	8.9
	0.50	"	16.0	17.8	1.6
	0.55	"	9.8	12.5	0.7
	0.60	"	3.2	3.2	0.1

Cells, 1×10^4/m*l*, were incubated with 4% cellulase Onozuka R-10 and 0.4% Macerozyme R-10 in 0.03 M phosphate buffer (pH 5.5) at 24°C. a, Cells were grown for 14 days; b, Cells were grown for 16 days.

concentrations more than 0.7 M and 0.55 M, respectively (Table 4). By the 24 hr-treatment, the protoplasts were disrupted.

The cell wall was started to be digested at the middle of the cells presumably at a position along the division suture. The digested area extended over the whole cells and naked protoplasts were formed. In this process, thread-formed chloroplasts were swollen, changed to spiral form, and then folded in the spherical protoplasts (Fig. 1, A–D). Several types of the protoplasts including the spiral chloroplasts were formed during the

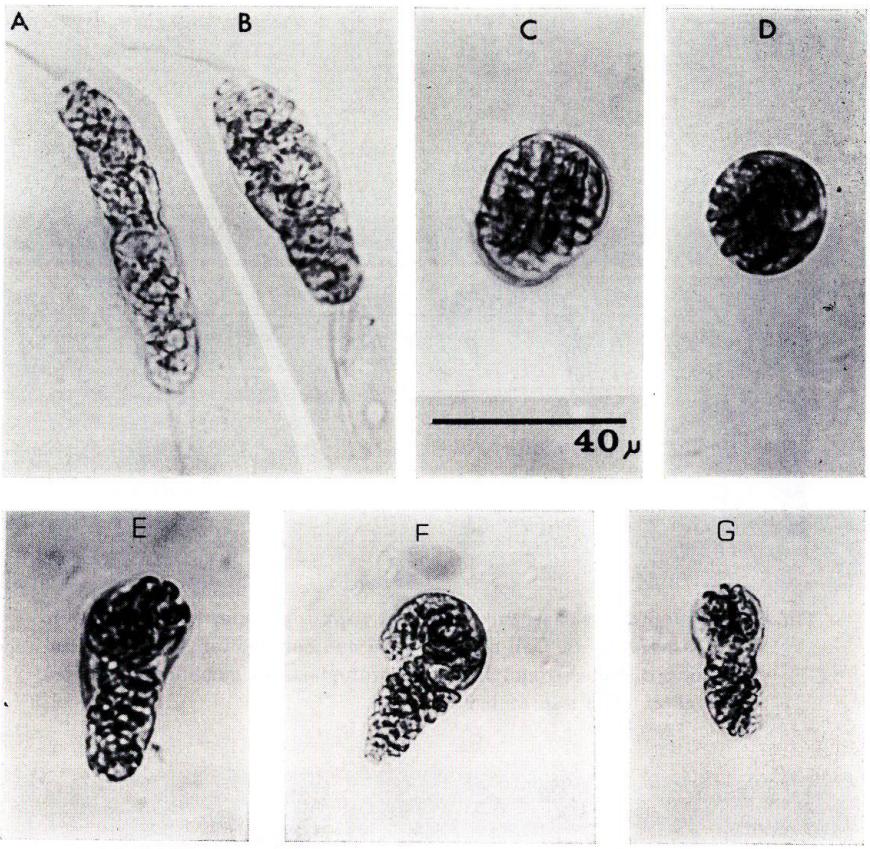

Fig. 1. Protoplast production from vegetatively growing mt^+-cells of heterothallic *Closterium*. The cells were treated with 4% cellulase and 0.5% Macerozyme in 0.03 M MES buffer (pH 5.5) in the presence of 0.5 M sorbitol at 24°C for 5 hr. A–D, Process of spherical protoplast production; E–G, Formation of spiral chloroplasts in course of protoplast production. The size of bar is 40 μm.

cell wall digestion (Fig. 1, D–F). The spirally folded chloroplasts were formed from the vegetatively growing cells of both the sexually opposite strains. During the mating process, the spiral folding of chloroplasts was not found in the protoplasts from paired gametes (Fig. 2) and in unpaired mt^+-cells (Fig. 3).

Protoplasts from C. acerosum

Protoplasts were also produced from the vegetatively growing cells though it was only a low efficiency. The yield with 4% cellulase Onozuka and 2% Macerozyme in 0.5 M sorbitol, at pH 5.8, at 25°C for 5 hr was only about

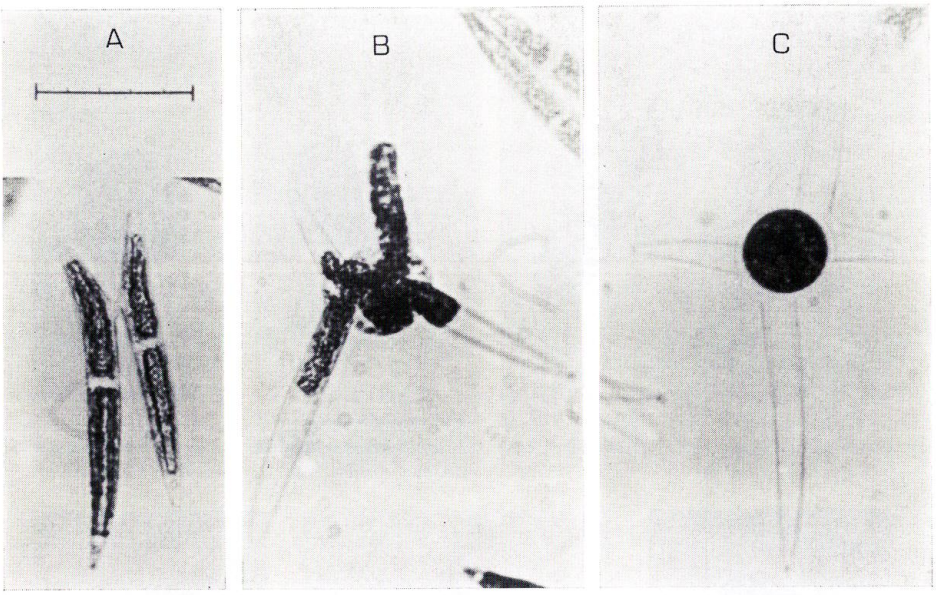

Fig. 2. Protoplast release and fusion in paired gametes of heterothallic *Closterium*. A, Cell-cell adhesion and conjugation papilla formation; B, Release and fusion of protoplasts of gametes; C, Naked zygote. The size of bar is 50 μm.

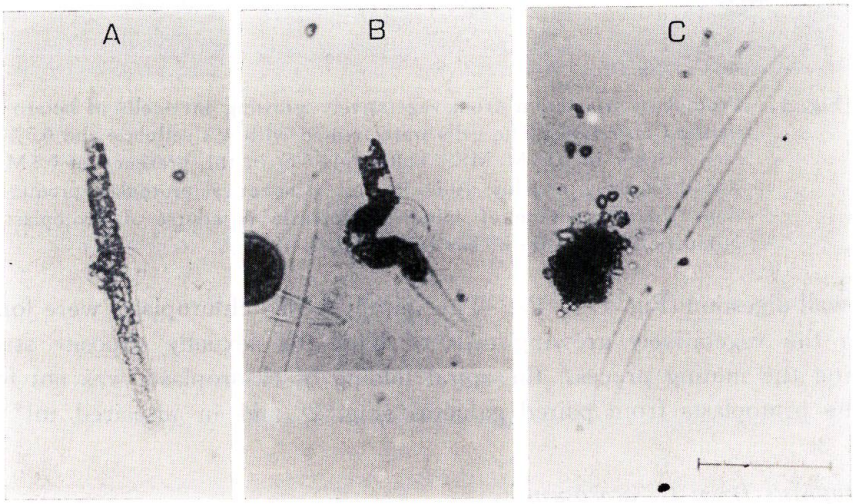

Fig. 3. Protoplast release and disruption in unpaired mt^+-cells during mating culture. A, Partial digestion of cell wall and formation of conjugation pappila-like swelling; B, Movement of protoplast toward the digested position; C, Protoplast released from the cell is in destruction. The size of bar is 50 μm.

8%. Profile of spherical protoplast production was as follows : the cell wall was partially digested and formed conjugation papilla-like swelling at a position along the division suture, and then naked protoplast moved into the swelling part was thrust out of the cells (Fig. 4, A–D). During the process, spirally

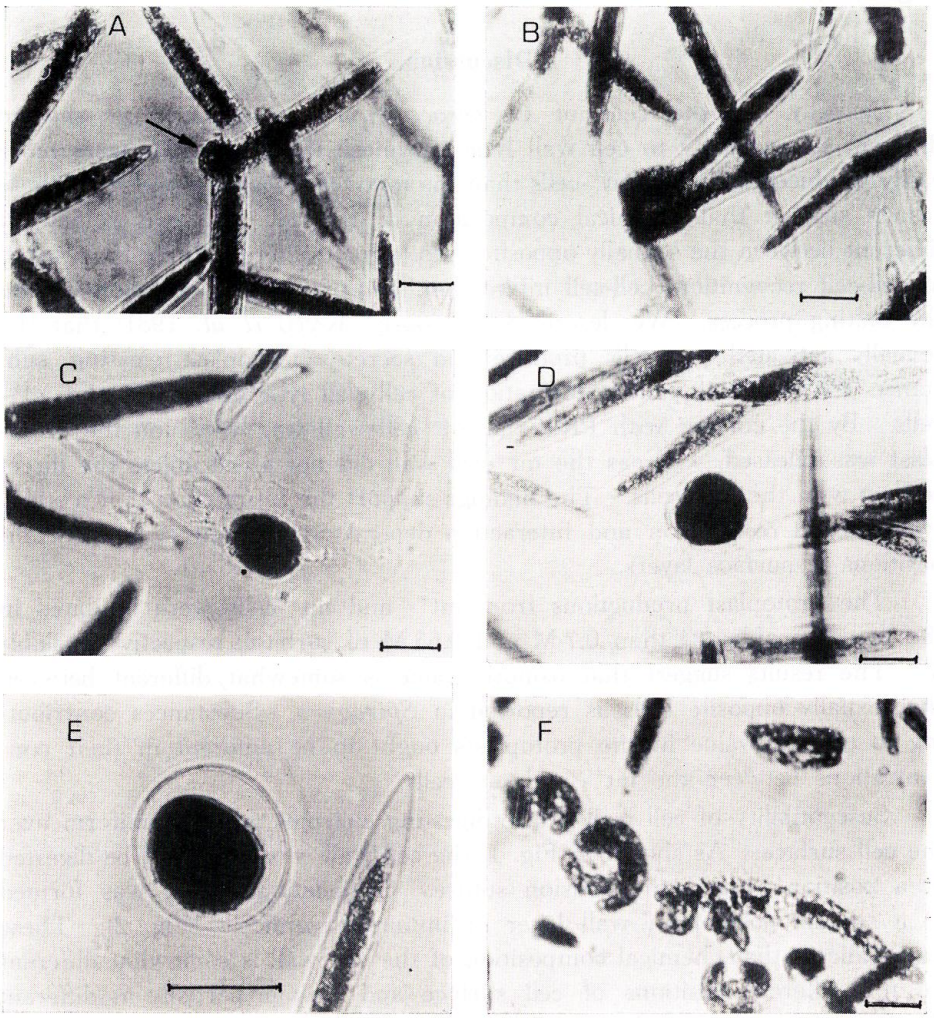

Fig. 4. Protoplast production from vegetatively growing cells of homothallic *Closterium*. The cells were treated with 4% cellulase and 2% Macerozyme, at pH 5.5, in the presence of 0.5 M sorbitol at 25°C for 5 hr. A, Partial digestion of cell wall and formation of conjugation pappila-like swelling (at arrow); B, Movement of protoplast toward the digested position; C, Release of protoplast; D and E, Regeneration of cell wall; F, Protoplasts produced by 24 hr-treatment. The size of bar is 100 μm.

folded chloroplasts was not found in the protoplasts. After 24 hr of the treatment, the cell wall was almost completely digested (Fig. 4, F). The spherical protoplasts were enclosed with the regenerated cell wall and came to the zygote-like cells in enzyme-free growth medium within 5 hr at 25°C (Fig. 4, E).

Discussion

Sexually opposite cells of *C. peracerosum-strigosum-littorale* showed different susceptibility to cell wall lytic enzymes: the protoplasts were more easily produced from the mt^+-cells than the mt^--cells (Tables 3 and 4). These results suggest that chemical composition of the cell wall was somewhat different between the sexually opposite cells. Such difference may be involved in cell-cell recognition, cell-cell interaction, and/or cell-cell adhesion during the mating process. We described previously (KATO *et al.* 1981) that the sexually activated mt^--cells produce and secrete "protoplast-releasing substance (PRS)" which induce formation of cell-wall lytic enzyme in the mt^+-cells. By the contact with PRS, the mt^+-cell wall was lysed and the protoplast was released, whereas the mt^--cell wall did not at all unless the direct contact with the mt^+-cells. The findings support the assumption that mating-type cell-cell recognition and interaction depend on different chemical compositions of surface layers.

The protoplast productions from mt^+- and mt^--cells were inhibited in the presence of more than 0.7 M and 0.55 M of sorbitol, respectively (Table 4). The results suggest that osmotic value is somewhat different between the sexually opposite cells as reported in *Spirogyra*. Substances contributing to osmotic value in the protoplasts ought to be different in their concentrations between the mt^+- and mt^--cells.

Susceptibility of cell wall to the digesting enzymes was not uniform over the cell surface. As shown in Fig. 1, the cell wall was started to be digested at a position around the division suture. Conjugation papilla was formed at a specific position of wall layer of immature semicells (Fig. 2). These data indicate that chemical composition of the cell wall is somewhat different on the different positions of cell surface and change partially at different developmental stages. Such differences were also shown in homothallic *Closterium acerosum*.

Using biologically active protoplasts, comparative studies on sexual and asexual cell fusions are in progress.

References

ADAMICH, M. and SWEENEY, B. M. 1976. The preparation and characterization of *Gonyaulas* spheroplasts. Planta **130**: 1–6.

CHARDARD, R. 1972. Production de protoplastes d'algues par un procédé physique. C. R. Acad. Sci. (Paris) **274**: 1015–1018.

GABRIEL, M. 1970. Formation, growth and regeneration of protoplasts of the green alga *Uronema gigas*. Protoplasma **70**: 135–138.

ICHIMURA, T. 1971. Sexual cell division and conjugation-papilla formation in sexual reproduction of *Closterium strigosum*. *In* Proc. 7th Internatl Seaweed Symp. Sapporo, pp. 208–214.

ITO, M. 1973. Studies on the behavior of meiotic protoplast. II. Induction of a high fusion frequency in protoplasts from liliaceous plants. Plant & Cell Physiol. **14**: 865–872.

KATO, A., OBOKATA, J. and SASAKI, K. 1982. Mating type interaction in *Closterium peracerosum-strigosum-littorale*: Mating induced protoplast release. Plant & Cell Physiol. **22**: 1215–1222.

KOMBRINK, E. Z. and WÖBER, G. 1980. Preparation of intact chloroplasts by chemically induced lysis of the green alga *Dunaliella marina*. Planta **149**: 123–129.

MARCHANT, H. J. and POWKE, L. E. 1977. Preparation, culture and regeneration of protoplasts from filamentous green algae. Can. J. Bot. **55**: 3080–3086.

MÜLLER, B. A., CALLOW, M. E. and EVANS, L. V. 1979. Preparation of protoplasts from green alga *Enteromorpha intestinalis* (L.) Link. Planta **147**: 174–177.

SASAKI, K., UENO, T. and SHIONO, K. 1976. Fundamental properties of uridine metabolism in *Closterium acerosum*. Jour. Fac. Sci., Hokkaido Univ., Ser. V (Botany) **10**: 157–168.

UENO, T. and SASAKI, K. 1978 a. Light dependency of mating process in *Closterium acerosum*. Plant & Cell Physiol. **19**: 245–252.

UENO, T. and SASAKI, K. 1978 b. Synchronous sexual cell division in L_{200} cells of *Closterium acerosum*. Jour. Fac. Sci., Hokkaido Univ., Ser. V (Botany) **11**: 218–224.