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Isolation of protoplasts from four species of brown algae

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Abstract. Preparation of protoplasts by the enzymatic method was investigated in four species of brown algae: *Endarachne binghamiae*, *Petalonia fascia*, *Sargassum duplicatum*, and *Undaria pinnatifida*. It was found that bovine serum albumin was required with blade material, with an optimum of 0.4%, but not with filament. A combination of 4% cellulase Onozuka RS and 2% macerozyme R-10 was effective for all parts of all species except *S. duplicatum* blade; for the latter, mollusk gastro-acetone extract powder was also required. The influence of EGTA pretreatment was variable. Positive effects were obtained only with material having a relatively large amount of cell wall (i.e. alginin content). With others, the effect was negative, no matter how short the treatment. Digestion at 20 °C optimized protoplast yield and viability. Variations in the *E. binghamiae* and *P. fascia* material collected on different dates, and the condition of the *S. duplicatum* culture also influenced protoplast yield. Seawater containing 0.8 M sorbitol was found to be optimum for the initial culture of protoplasts, and 2,4-D had a stimulating influence on protoplast survival, with an optimum concentration of 10 ppm.

Keywords: Brown algae; Isolation; Protoplast.

Introduction

Protoplasts are a tool used in many fields, such as physiology, biochemistry, somatic hybridization, molecular biology, and genetic engineering. Improved protoplast isolation techniques have made possible the utilization of protoplasts from an increasing number of plants (Eriksson, 1986). Protoplast isolation from seaweed had been investigated, but progress lagged behind work with higher plants. This is due mainly to the uniqueness of the algal cell wall, in particular that of brown and red algae, which is not completely removed during enzymatic digestion. In recent years, an enzyme from marine mollusks and microorganisms has led to a breakthrough in the digestion of algal cell wall material (Butler et al., 1989; Kloareg et al., 1989), and thus the preparation of their protoplasts. There have also been many improvements in the preparation and culture of protoplasts which we will consider (Butler et al., 1990; Polne-Fuller and Gibor, 1987; Saga, 1984). Though there are only nine species (seven genera) of brown algae with which there has been successful protoplast isolation (cf. Butler et al., 1990; Tokuda and Kawashima, 1988; Uchida and Arima, 1992), many more are of interest. For this study, we chose four species of brown algae — three native species, *Endarachne binghamiae*, *Petalonia fascia*, and *Sargassum duplicatum*, and one exotic species, *Undaria pinnatifida*, because they have different tissue structures and environmental adaptabilities. We compare the following aspects of protoplast isolation:

Finding a suitable enzyme combination for cell wall digestion, using commercial enzyme preparations.

Reducing damage to protoplasts caused by contaminated protease by adding protease inhibitor or protein substrate to the digestion mixture.

Treating alginin in brown algae with a chelator prior to enzymatic digestion to facilitate protoplast liberation.

Finding an enzymatic digestion temperature that optimizes yield and viability.

Selecting suitable material with relatively simple cell walls to ease enzymatic digestion.

Finding an optimum osmotic pressure in the initial culture medium.

Improving initial survival by adding plant-growth regulator to stimulate protoplast cell wall synthesis.

Materials and Methods

Collection and Culture of Algae

Four species of brown algae were investigated using blade and filament material. Tissue of *Sargassum duplicatum* was taken directly from laboratory culture; the thalli were cultured in Provasoli's (1968) enriched seawater in 1 liter flasks or in 40 x 20 x 35 cm aquaria. Cultering was carried out in a walk-in growth room

with the temperature maintained at 20 ± 0.5 °C and illuminated by fluorescent lamps set at 42 μE m^{-2} s^{-1} in a light-dark cycle of 12h:12h. Constant agitation was provided using filtered compressed air. The other materials were collected in northern Taiwan during their growth season (Jan-Apr) in 1992. *Endarachne binghamiae* and *Petalonia fascia* were collected from the intertidal area near Keelung, and *Undaria pinnatifida* from the coastal abalone-culture pond at Makung, Taipei County. The algal thalli were wrapped with a seawater-moistened paper towel and immediately transported to the laboratory in a cooler. The thalli were cleaned of epiphytes, dipped in 1% betadine solution for 5–10 min, and washed several times with sterilized seawater just prior to use.

For preparation of filamentous material, matured thalli of both *E. binghamiae* and *P. fascia* were chosen from the field-collected samples. The thalli were exposed overnight to air in a 9 x 2 cm dish under darkness and then submerged in sterilized seawater. The zooids thus stimulated to release a moment later were collected in a water drop through the agency of their phototacticity. The concentrated zooids were dispensed at the darker end of a dish filled with fresh sterilized seawater and collected again as before. The zooids were cultured in 50 ml flasks under the same conditions as for thallus culture. They stopped swimming within a few hours, began to divide in 2–3 days, and continued developing into filaments which were then scraped off the flask and cultured for subsequent use.

Preparation of Enzyme Solution

The enzymes used in this study are: cellulas Onozuka RS (O), Macerozyme (M) (Yakult Co.), abalone acetone powder (A), and limpet acetone powder (L) (Sigma Co.). The preparation was divided into the following steps:

Solution A: dissolved 0.9 g of limpet acetone powder or abalone acetone powder in 10 ml phosphate buffer (pH 6) by stirring at 4 °C overnight. The extract was poured into a 10 ml centrifugation tube, followed by centrifugation at 15000 x *g* at 2 °C for 40 min. The supernantant was sterilized by filtration through 2.0, 0.8, 0.45, and 0.22 μm mesh filters and then stored at -15 °C.

Solution B: dissolved 0.6 g cellulase and 0.3 g macerozyme into 10 ml 2.4 M sorbitol, based on phosphate buffer (pH 6). The extract was centrifuged and filter-sterilized as for solution A and stored at -15 °C.

Solution C: dissolved bovine serum albumin (BSA) (Sigma Co.) in 1.2 M sorbitol solution, based on phosphate buffer (pH 6), to final concentrations of 0, 0.4, 1.2, and 3%. The solutions were sterilized by filtering through 0.22 μm membrane filter and stored at 4 °C.

Solution D: dissolved 3.482 g of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) (Sigma Co.) in 10 ml 95% ethyl alcohol to make a 2 M solution and sterilized it as for solution C. The stock was further diluted to 1:100 before use.

The proportions of the four parts depended on the experiment. In studying the influence of BSA and PMSF on protoplast yield, solutions A, B, and C were combined in a ratio of 1:1:1, using each of the BSA solutions (0, 0.4, 1.2, and 3%). Solutions A, B, and C were also combined in the same ratio in another group with 10 mM PMSF (solution D) added to the 0 and 0.4% BSA-based mixture. In the subsequent experiment for enzyme-composition effect on protoplast yield, solutions A, B, and C were combined 1:1:1 with BSA solutions of 0 and 0.4% only. All the enzymatic digestions were run with 1.5 ml of enzyme mixture, in triplicate, in a six-well plate (Falcon). During digestion, the plate was mounted on a 60 RPM rotatary shaker in darkness, and incubated at various temperatures between 15–30 °C.

Preparation of EGTA Solution

Ethylene glycol-bis(amino ethyl ether)-N, N-tetracetic acid (EGTA, Sigma Co.) solution was prepared according to Kloareg and Quatrano (1987). Deionized water was used to make artificial seawater containing 450 mM NaCl, 100 mM $MgCl_2 \cdot 6H_2O$, and 20 mM KCl, based on which 20 mM EGTA solution was prepared and adjusted to pH 6.5.

Preparation of Protoplasts

Algal materials (blade/filaments) for the four experimental species were treated with 20 mM EGTA solution for 0 (control) to 4 min before enzymatic digestion. After being washed three times with sterile seawater, algal material was cut into pieces (approximately 1.0 mm^2) and 0.05 g wet weight was put into each well of a six-well plate, followed by adding 1.5 ml of one of the enzyme mixtures. The enzyme digestion took place on a 60 RPM rotary shaker in darkness in a 15–30 °C incubator.

The digestion time for substantial liberation of protoplasts from *E. binghamiae, P. fascia,* and *U. pinnatifida* was 2 and 3–5 h for filament and blade respectively, while that for blade of *S. duplicatum* was 12–19 h. After harvesting, the digested liquid was filtered through 59 μm and 30 μm nylon mesh to remove debris, followed by centrifugal washing with 8.5 ml of 1.2 M sorbitol solution at 200 x *g* for 5 min. The supernatant liquid was discarded. The pellet was resuspended in 0.8 M sorbitol-seawater solution. The protoplast solution was sampled, and counted with a hemacytometer under a microscope (Nikon Diaphot-TMD). Protoplast yield was expressed as number of protoplasts obtained per gram wet weight. Viability was examined by staining with Evans' blue (Evans and Bravo, 1983) for 5 min and counting under a microscope as above. The value was expressed as yield. The statistical analysis was processed by the SAS Package on a PC using one-way ANOVA.

Table 1. Influence of BSA and PMSF on protoplast yield of *Endarachne binghamiae* prepared by the enzymatic method.

Algal phase	Enzyme combination[1]	Additive[2]	Yield[3]
Thallus	OML	0.4% BSA	$(3.75^{a} \pm 0.05)10^{8}$
		1.2% BSA	$(3.05^{c} \pm 0.10)10^{8}$
		3.0% BSA	$(3.37^{b} \pm 0.15)10^{8}$
		0.2 M PMSF	$(2.62^{d} \pm 0.08)10^{8}$
		0.4% BSA + 0.2 M PMSF	$(3.35^{b} \pm 0.17)10^{8}$
Filament	OM	4% BSA	$(0.73^{a} +0.32)10^{8}$
		0	$(0.68^{a} \pm 0.19)10^{8}$

[1]**O**) Onozuka RS; **M**) macerozyme; **L**) limpet acetone powder.
[2]**BSA**) bovine serum albumin; **PMSF**) phenyl-methyl sulphonylfluoride.
[3]**a**,**b**,**c**, and **d** represent the Duncan groups, in the sequence: a > b > c > d ($P < 0.05$).

Culture of Protoplasts

In searching for optimum osmotic conditions for culture, 0.5 ml of the protoplast concentrate was dispensed in 10 ml capped centrifugal tubes, each containing 9.5 ml of sorbitol-adjusted seawater (pH = 8) with osmolality ranging from 0–650 milliosmols Kg^{-1} (adjusted by 0–1.6 M sorbitol). The tubes were then mounted on a rotory plate and incubated at 20 °C, rotating at 20 RPM in darkness. Changes in protoplast number were monitored at 0, 10, and 24 h after incubation. The osmotic concentration causing the least stress was adopted for the subsequent experiments.

In further culture of protoplast, the culture media was enriched with the plant growth regulator 2,4-D at concentrations of 0–10 ppm to study its effect on protoplast survival. The experiment was conducted in a 50 ml flask containing 20 ml of culture media. Protoplasts were first left for one week to settle firmly on the bottom of the flask, during which time the osmolality of the culture media was gradually lowered from 1820 to 1001 milliosmols Kg^{-1} (0.8 M sorbitol to plain seawater). The 2,4-D enriched media was thereafter used to replace seawater and was replenished every other day. Changes in the number of protoplasts were monitored under an inverted microscope as above.

Result

The Effect of BSA and/or PMSF on the Protoplast Yield in *E. binghamiae*

Only blade tissue was used in the experiment with *E. binghamiae*. Various protoplast yields were found with the addition of either BSA or PMSF, and none was obtained with the control (Table 1). The highest yield was obtained at 0.4% BSA (1.23 and 1.11 times that with 1.2% and 3% BSA, respectively). Treatment with PMSF alone gave the lowest yield, only 70% of that with 0.4% BSA. The combination of 0.02 mM PMSF and 0.4% BSA produced a yield near that with 3% BSA, and therefore the 0.4% BSA treatment was adopted for the subsequent experiments.

The Effect of Various Enzyme Combinations on Protoplast Yield

The blade and filament material of four species of brown algae were digested enzymatically for protoplast preparation using various enzyme combinations with/without addition of BSA (Table 2). No protoplasts were obtained from algal blade of any species without BSA. Generally, only two hours were required for substantial liberation of protoplasts from filaments of *E. binghamiae* and *P. fascia*, three hours from their blade, three to five hours for *U. pinnatifida* blade, and as long as 12–16 hours for *S. duplicatum* blade. As shown in Table 2, the yields can be listed in decreasing order of OML>OMA>OM except with *P. fascia*, in which OM produced slightly better yield than did OMA. The inferiority of the OM combination was more clearly manifested in *U. pinnatifida* (significantly less) and *S. duplicatum* (absolutely nothing). In the digestion of *Sargassum* cell wall OM was ineffective. Adding acetone-extracted powder of abalone or limpet caused a substantial liberation of protoplasts, but about five-times-longer incubation was required. *U. pinnatifida* exhibited cell wall digestion closer to that of *E. binghamiae* and *P. fascia*, but with less yield.

The Effect of EGTA Pretreatment on the Yield of Pprotoplasts

As shown in Table 3, EGTA pretreatment enhanced protoplast release only in very few cases; normally no enhancement was found. On the contrary, the yield was reduced further as treatment duration increased. For example, yield from *E. binghamiae* blade treated with 20 mM EGTA for only 30 s was reduced to 0.46% of that of untreated material. Similar results were obtained with *P. fascia* and *U. pinnatifida*. With *S. duplicatum*, the treatment produced quite varied results. Material from flask culture could be treated with EGTA for more than 3 min to obtain a significant yield, while few were obtained with the control or the shorter-duration treatment. Using material from aquarium culture, however, resulted in a yield one order of magnitude larger without any EGTA pretreatment.

Table 2. The influence of enzyme combination and BSA on protoplast yield of brown algae.

Algal species	Phase	Enzyme comb.[1]	BSA[2]	Time of incubation	Yield[3]
Endarachne binghamiae	thallus	OM	W	3	$(0.42^{c} \pm 0.16)10^{8}$
			N	3	0
		OML	W	3	$(1.92^{a} \pm 0.28)10^{8}$
			N	3	0
		OMA	W	3	$(1.10^{b} \pm 0.09)10^{8}$
			N	3	0
	filament	OM	W	2	$(0.73^{a} \pm 0.24)10^{8}$
			N	2	$(0.68^{a} \pm 0.18)10^{7}$
Petalonia fascia	thallus	OM	W	3	$(2.17^{b} +0.10)10^{7}$
			N	3	0
		OML	W	3	$(3.07^{a} \pm 0.28)10^{8}$
			N	3	0
		OMA	W	3	$(1.67^{c} \pm 0.21)10^{8}$
			N	3	0
	filament	OM	W	2	—[4]
			N	2	—[4]
Sargassum duplicatum	thallus	OM	W	12–19	0^{c}
			N	12–19	0
		OML	W	12–19	$(9.71^{a} \pm 0.50)10^{5}$
			N	12–19	0
		OMA	W	12–19	$(8.40^{b} \pm 0.95)10^{5}$
			N	12–19	0
Undaria pinnatifida	thallus	OM	W	3–5	$(0.77^{c} \pm 0.22)10^{6}$
			N	3–5	0
		OML	W	3–5	$(8.33^{a} \pm 1.15)10^{6}$
			N	3–5	0
		OMA	W	3–5	$(6.67^{b} \pm 0.58)10^{6}$
			N	3–5	0

[1] **O**, **M**, and **L** as in Table 1; **A**) abalone acetone powder.
[2] **BSA**) as in Table 1; **W**) with, **N**) without.
[3] Expressed as number of protoplasts per gram fresh weight of algal thallus; **a, b, c,** and **d** represent the Duncan groups, compared within each species, in the sequence a > b > c > d ($P < 0.05$).
[4] No data (due to insufficient filament material).

The Influence of Temperature on Protoplast Yield and Viability

The influence of temperature on protoplast yield varied with algal species (Table 4). The optimum temperature, common to all the species but *E. binghamiae*, was 20 °C. With *E. binghamiae*, protoplast yield increased as temperature was raised from 15 to 30 °C. The yield at 15 °C was only half of that at 30 °C. The highest viability, however, was 36% at 20 °C, compared to 32% at 15 °C and 20% at 30 °C. With *P. fascia*, the best yield was found at 20 °C. No significant difference in yield was shown at other temperatures. The protoplast was most viable at 15 and 20 °C. With *S. duplicatum*, the best yield was found at 20 °C. Data for viability was unavailable because very few protoplasts remained survived staining with Evans' blue. With *U. pinnatifida*, protoplast yield decreased drastically from 20 to 30 °C; only one-tenth that of the 20 °C group was obtained at 30 °C.

The Influence of Collection date on Protoplast yield

Variation in protoplast yield among *E. binghamiae* and *P. fascia* material collected on different dates was obvious (Table 5). With *E. binghamiae*, higher yield was obtained with February plants (2.0–6.7 $x10^{8}$ per gram fresh weight) than with March plants (0.28–1.13 $x\ 10^{8}$ per gram fresh weight). Two types of protoplasts could be distinguished, large ones of 10–15 μm diameter, and small one of 3–5 μm diameter. The proportion of each type also varied with plant material; those collected earlier had a higher proportion of large ones (0.01–0.12%) than did those collected later (0.01%). Similar results were found with *P. fascia* in early and late March.

Table 3. The influence of duration of EGTA pretreatment on protoplast yield of four brown algae.

Algal species	Enzyme combination[1]	Duration of EGTA pretreatment[2]	Yield[3]
Endarachne binghamiae	OMA	2 min	$(2.77^{b} \pm 0.41)10^{8}$
		0	$(4.06^{a} \pm 0.66)10^{8}$
Petalonia fascia	OML	30 s	$(3.25^{a} \pm 0.35)10^{8}$
		60 s	$(1.40^{b} \pm 0.36)10^{8}$
		0	$(3.07^{a} \pm 0.29)10^{8}$
	OMA	30 s	$(0.96^{b} \pm 0.05)10^{5}$
		60 s	$(0.48^{c} \pm 0.03)10^{8}$
		0	$(1.69^{a} \pm 0.16)10^{8}$
Sargassum duplicatum			
Aquarium culture :	OML	0	$(2.78^{a} \pm 0.29)10^{5}$
		30 s	$(1.29^{b} \pm 0.17)10^{5}$
		1 min	$(1.04^{c} \pm 0.17)10^{5}$
		2 min	$(0.71^{d} \pm 0.09)10^{5}$
		3 min	$(0.41^{e} \pm 0.12)10^{5}$
		4 min	$(0.29^{f} \pm 0.12)10^{5}$
Flask culture :	OML	0	Very few
		30 s	Very few
		1 min	Very few
		2 min	Very few
		3 min	$(1.80^{a} \pm 0.04)10^{4}$
		4 min	$(4.40^{b} \pm 0.70)10^{4}$
Undaria pinnatifida	OML	30 s	$(0.90^{ab} \pm 1.78)10^{6}$
		1 min	$(0.73^{bc} \pm 0.13)10^{6}$
		2 min	$(0.65^{cd} \pm 0.09)10^{6}$
		3 min	$(0.48^{d} \pm 1.11)10^{6}$
		0	$(1.08^{a} \pm 1.56)10^{6}$

[1] **O**, **M**, **L**, and **A** as in Tables 1 and 2.
[2] **EGTA**) ethylene glycol-bis(amino ethyl ether)-N,N'-tetracetic acid.
[3] Expressed as number of protoplasts per gram fresh weight of algal thallus; **a**, **b**, **c**, **d**, **e**, and **f** represent the Duncan groups, compared within each species, in the sequence a > b > c > d > e > f ($P < 0.05$).

The Influence of Osmotic Concentration of Incubation Media on the Survival of Protoplasts after Isolation

The three species other than *S. duplicatum* were used in the osmotic adaptability test. All three species exhibited a decreasing population at every osmotic concentration (Table 6). Although the trend varied with different osmotic environments as well as with algal species, the osmotic concentration producing the slowest population decrease was 0.8 M sorbitol in seawater (1791 millios-mols kg^{-1}). With both *E. binghamiae* and *P. fascia*, 90% survived at the 5th hour after the incubation, compared to 76% of *U. pinnatifida*. The former two, however, showed a more rapid reduction as incubation progressed. All three populations exhibited relatively close survival rates at the end of the experiment.

The Influence of 2,4-D on the Survival of Protoplasts

Protoplasts of the four species were cultured in the seawater medium producing the least osmotic stress (0.8 M sorbitol in seawater) after isolation. Those of *S. duplicatum* and *U. pinnatifida* suffered great mortality during the first few days of culture and continued to die off to near non-existence within one week, whereas approximately 4% of those from the other two species survived the great mortality period. The protoplasts with greater survival rate were used in the studies of plant growth regulator (2,4-D) effects. As shown in Table 7, the presence of 2,4-D reduced the initial mortality of protoplast population to some extent at every concentration; the effect was more obvious as the concentration was increased from 2.5 to 10 ppm.

Table 4. The influence of temperature on protoplast yield and viability of four brown algae.

Algal species	Temperature	Protoplast Yield[1]	Viability (%)
Endarachne binghamiae	15 °C	$(3.55^{d} \pm 0.34)10^{8}$	32.63
	20 °C	$(4.65^{c} \pm 0.24)10^{8}$	35.94
	25 °C	$(5.70^{b} \pm 0.55)10^{8}$	20.59
	30 °C	$(7.15^{a} \pm 0.37)10^{8}$	16.22
Petalonia fascia	15 °C	$(3.07^{b} \pm 0.24)10^{8}$	54.48
	20 °C	$(3.73^{a} \pm 0.11)10^{8}$	54.26
	25 °C	$(3.05^{b} \pm 0.13)10^{8}$	35.02
	30 °C	$(3.02^{b} \pm 0.30)10^{8}$	28.00
Sargassum duplicatum	15 °C	$(2.22^{bc} \pm 0.56)10^{5}$	—[2]
	20 °C	$(3.89^{a} \pm 0.56)10^{5}$	—
	25 °C	$(1.85^{c} \pm 0.32)10^{5}$	—
	30 °C	$(3.15^{ab} \pm 0.85)10^{5}$	—
Undaria pinnatifida	15 °C	$(2.22^{b} \pm 0.22)10^{6}$	45.69
	20 °C	$(3.63^{a} \pm 0.71)10^{6}$	60.00
	25 °C	$(1.37^{c} \pm 0.23)10^{6}$	0
	30 °C	$(0.37^{d} \pm 0.13)10^{6}$	0

[1] Expressed as number of protoplasts per gram fresh weight of algal thallus;
a, b, c, and **d** represent the Duncan groups, compared within each species, in the sequence a > b > c > d ($P < 0.05$).
[2] No data (due to insufficient thallus material).

Table 5. Variation of protoplast yield with thallus of *Endarachne binghamiae* and *Petalonia fascia* collected on different dates

Algal species	Date of field collection	Size of protoplasts[2]	Yield[1]
Endarachne binghamiae	92.1.13	S	$(4.92^{b} \pm 1.06)10^{8}$
		L	$(0.58^{a} \pm 0.14)10^{8}$
	92.1.15	S	$(6.72^{a} \pm 0.02)10^{8}$
		L	$(0.84^{b} \pm 0.02)10^{8}$
	92.1.18	S	$(2.35^{c} \pm 0.06)10^{8}$
		L	0.00^{c}
	92.3.10	S	$(1.10^{d} \pm 0.09)10^{8}$
		L	0.00^{c}
	92.3.17	S	$(0.28^{e} \pm 0.03)10^{8}$
		L	0.00^{c}
	92.3.20	S	$(0.12^{e} \pm 0.02)10^{8}$
		L	0.00^{c}
Petalonia fascia	92.3.05		$(7.00^{a} \pm 1.26)10^{8}$
	92.3.11		$(3.07^{b} \pm 0.22)10^{8}$
	92.4.07		$2(3.73^{c} \pm 0.11)10^{8}$

[1] Expressed as number of protoplasts per gram fresh weight of algal thallus;
a, b, c, and **d** represent the Duncan groups, compared within each species, in the sequence a > b > c > d ($P < 0.05$).
[2] **S**) small (3–5 μm); **L**) large (10–15 μm); size was not differentiated in the enumeration of *P. fascia* protoplasts.

Discussion

It was found that, with the exception of *S. duplicatum*, all of the material could be digested with the simplest enzyme combination, implying a low alginin content in the cell wall. The paucity of alginin content was manifested more in the cell wall of algal filament, since adding mollusk enzyme reduced rather than increased protoplast yield. This is thought to be due to the interferring effect of extra enzyme activity. Another character of protoplast preparation with algal filament was the absence of a need for protein substrate or

Table 6. The survival rate of protoplasts of three species of brown algae under various osmotic conditions.[1]

Algal species	Time after treatment (h)	Concentration of sorbitol in seawater (M)				
		0	0.4	0.8	1.2	1.6
Endarachne binghamiae	5	29.41	32.21	95.03	73.91	47.68
	10	6.94	21.56	88.51	63.66	27.95
	24	1.67	2.14	37.85	34.12	5.43
Petalonia fascia	5	51.14	59.07	94.34	77.08	66.37
	10	9.32	23.72	72.01	51.15	39.82
	24	2.65	3.25	35.84	28.33	26.55
Undaria pinnatifida	5	44.20	56.91	76.09	52.84	42.39
	10	29.35	43.08	67.39	37.80	26.09
	24	13.04	18.29	36.96	17.07	11.96

[1] Data for *Sargassum duplicatum* is not expressed due to low viability.

Table 7. The influence of 2,4-D on the survival of protoplasts from *Endarachne binghamiae* and *Petalonia fascia* over eight days of incubation.[1]

Algal species	Concentration (mg/l)				
	day	0	2.5	5.0	10.0
Endarachne binghamiae	0	11.43	12.55	11.62	12.52
	4	5.63	8.14	8.93	12.16
	6	4.77	7.84	8.63	10.90
	8	4.00	7.53	8.38	10.68
Petalonia fascia	0	10.76	11.03	10.80	10.80
	4	5.36	6.24	9.43	10.07
	6	5.24	5.93	8.44	9.70
	8	3.98	5.59	6.89	8.51

[1] Mean of fifteen 200X microscope-field observations; expressed as percentage of initial inoculation.

inhibitor. This could be ascribed to its simple cell wall composition, allowing a significantly shorter digestion time, and thus harvesting before being adversely affected by the protease. As cell wall became more complex, more time and enzyme activity were required. As a result, more contaminating enzymes would be involved , making BSA or PMSF addition indispensible. The application rate is therefore dependent on the amount of contaminating enzyme. Both to little and too much BSA depressed yield; in this study, 0.4% of BSA was suitable. The yield reduction in the latter instance is attributable to a blocking of protease function. A certain amount of protease activity is considered helpful in the digestion of cell wall (Butler et al., 1990). The toxicity of the protein inhibitor, PMSF, is thought to be responsible for its being inferior to the protein substrate, BSA, for preparing protoplasts. Comparable results were obtained by its application to higher plants (Fitzsimons and Weyer, 1985). Furthermore, its relative insolubility in water also make it relatively inapplicable in protoplast preparation. For brown algae with relatively simple cell wall composition, such as *E. binghamiae* and *P. fascia*, mollusk acetone extract powder (A and L) exhibited sufficient digestion capability. For material with higher alginin content, more such enzyme would be required. Nevertheless, the same application rate of the extract powders also works well in the preparation of protoplast from *U. pinnatifida* and *S. duplicatum*. The results obtained were comparable to those reported by Tokuda and Kawashima (1987) and Fisher and Gibor (1985). Although the reason why limpet acetone powder is superior to that of abalone is still unknown, the convenience of preparing bacteria-free enzyme solution by membrane filtration and its lower cost made the limpet powder the preferred material.

As a supplementary process prior to enzymatic digestion, treatment of alginin with a chelator, resulting in the breakage of the calcium bridge between polymer chains and leading to dissolution of alginin, could facilitate protoplast liberation. Examples of use of the chelator included some brown algae (Butler et al., 1989; Davison and Polne-Fuller, 1990; Uchida and Arima, 1992). Since

alginin content in cell wall is variable, optimal treatment at suitable concentration for each material may vary correspondingly. For most of the materials used in this study, even short treatment reduced the yield. Extreme damage occured with algal filaments of *E. binghamiae* and *P. fascia*, in which all cells lost their turgor pressure in 20 s (unpublished data). Chelation of calcium ions by EGTA was assumed to cause the loss of membrane integrity in those algae with less alginin content. For those materials with moderate or more alginin content, like *S. duplicatum*, the yield obtained after EGTA pretreatment varied with the culture conditions. Alginin was assumed to accumulate more in flask-culture plants than in aquarium culture plants. Nitrogen deficiency would favor secondary product synthesis, including cell wall material, as described by some workers (Indergaard et al., 1978; DeBoer, 1979).

Temperature may play a major role in digestion. It may influence protoplast yield, either through its influence on enzyme activity or by changing protoplast viability. From a chemical standpoint, the optimal temperature for the three major enzymes is higher than 30 °C. This temperature, however, exceeds the range suitable for all protoplasts, especially those of temperate species, such as *U. pinnatifida*. Protoplasts from the latter totally lost their turgidity when digested at 30 °C, while with subtropical species, such as *E. binghamiae* and *P. fascia*, the effects of over-temperature are seen not in the yield but in the viability. The adaptability of seaweed in natural environments was roughly reflected in their protoplast preparation.

Besides Evans' blue, fluorescin diacetate (FDA) has been tried in the vital staining, but it is not as discriminative. The staining efficiency of various reagents has been evaluated by several workers with different categories of algae; the results are inconsistent. Kloareg and Quatrano (1987) reported that FDA is applicable to the vital staining of some brown algal protoplasts, while Saga et al. (1989) do not recommend it.

In addition to that experienced from *S. duplicatum* with respect to EGTA pretreatment, the material effect on yield was also found from both *E. binghamiae* and *P. fascia* of

The difference in yield associated with different collection dates, that is, the lengthening of digestion time and the shift in the proportion of the large and the small protplasts can be correlated with the accumulation of cell wall material and the sporulation of algal blade as it matures during period of early to late spring, respectively.

Very few protoplasts can grow without a supplement of plant-growth regulator in the culture media (Eriksson, 1985). The formation of cell wall is stimulated in the presence of plant growth regulator (Franz and Blaschek, 1985). This may illustrate the alleviation of initial mortality by use of the latter in protoplast culture.

All algal species used in this study had the best survival rate in seawater media supplemented with 0.8 M sorbitol, a value close to our previous result (Chen and Chen, 1990), implying that they have adapted to similar salinity. The great mortality of protoplasts immediately following isolation has been experienced with many species, even if a suitable osmotic environment was provided. Critically speaking, the osmolality of the media used in this study might not be optimum for the protoplast. It might also be partly to blame for both the defective protoplasts during preparation and the toxicity of the osmoticum (unpublished data). Further work on this is required.

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四種褐藻原生質體之分離

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四種褐藻包括小海帶及點葉藻重緣葉馬尾藻及裙帶菜葉狀體原生質體之製備，需有 BSA 之參與始能獲得完整之原生質體，小海帶及點葉藻絲狀體原生質體之製備則不需；而 BSA 濃度以 0.4% BSA 效果最佳。以 4% 纖維酶 (Yakult Co.), 2% 軟化酶 (Yakult Co.) 合併作用可酶解小海帶及點葉藻之葉狀體及絲狀體，以及裙帶菜之薄壁葉狀體（產率稍低）。重緣葉馬尾藻則需另加鮑魚或笠貝丙酮萃取粉末，始能獲得相當之產率。 EGTA 之前處理效果僅對細胞壁複雜（藻膠含量較多）之葉體原生質體之製備有促進作用，而其他葉體或絲狀體則無促進。解溫度影響其產率及活性，而以 20°C 之溫度爲佳。不同時期採集之小海帶及點葉藻以及不同培養條件之重緣葉馬尾藻材料亦影響產率。 0.8 M 山梨醇海水溶液適合原生質體之初期培養。 2,4-D 對小海帶及點葉藻葉體原生質體之活存，有促進作用。

關鍵詞：分離；原生質體；褐藻。