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Protoplast production from *Porphyra linearis* using a simplified agarase procedure capable of commercial application†

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Abstract

Abalone enzymes, Cellulase R-10, Macerozyme and agarase from *Pseudomonas atlantica* were tested for activity on agarose, cellulose, xylan, the cell wall matrix and porphyran isolated from *Porphyra linearis*. Agarase, and to a lesser extent Macerozyme, digested both agarose and porphyran. Abalone enzymes and Cellulase R-10 reacted only weakly with porphyran. A simple standardized protocol for making protoplasts from *Porphyra linearis* was developed using 0.025% agarase in seawater without added organic osmoticants. Protoplasts prepared with agarase remained viable for at least 24 h in the digestion medium. Regeneration of the protoplasts followed the normal pattern for this species. Agarase can be used to obtain large number of protoplasts which could substitute for conchospores in seeding nets for the aquaculture of *P. linearis*

Introduction

Porphyra linearis Grev. has been reported to be superior in texture, colour and taste to other commercially available cultivated species of nori (McLachlan *et al.*, 1972). Nevertheless, this species has yet to be developed commercially for nori production. Development of technology for the experimental production of hoshi-nori directly from cell suspension cultures derived from protoplasts of *P. linearis* has been described (Chen *et al.*, 1990); however, the acceptance by consumers of nori produced in this unconventional manner has yet to be demonstrated. Although conchospores of *P. linearis* have been observed (Bird *et al.*, 1972; Bird, 1973), we find that the conchospore discharge rate from conchocelis *in vitro* is low and unpredictable. Conventional aquaculture of this species in the sea is unlikely until a more convenient method is developed to ensure a supply of young plantlets. An alternative strategy in which protoplasts are substituted for conchospores has been used to produce *P. haitanensis* 'seed' for aquaculture (Tseng & Wang, 1991).

Although enzyme mixtures successfully release protoplasts from gametophytic fronds of *Porphyra*, the relationship between the enzyme activities and the cell wall digestion of *Porphyra* fronds has received little study (Liu *et al.*, 1984; Mizukami *et al.*, 1992, 1993). Without such information, the preparation of enzyme cocktails that consistently promote a high yield of healthy protoplasts from species of *Porphyra* is rendered difficult.

Currently, we report that five enzyme preparations including partially purified agarase extracted from *Pseudomonas atlantica* showed activity with various substrates and cell wall constituents. A simple routine procedure based on agarase was developed for the isolation of protoplasts from gametophytic fronds of *P. linearis*. This procedure can be scaled up and standardized for *P. linearis* aquaculture.

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Materials and methods

Algal tissue preparation

Porphyra linearis fronds were collected in the upper littoral zone at Peggy's Cove, Nova Scotia, and then cut into small pieces (area<0.5 m^2) prior to enzyme digestion.

Enzyme extraction and preparation

The enzymes used were either commercially available or extracted in the laboratory. Agarase was prepared from *Pseudomonas atlantica* grown in shake culture for 16 h at 25 °C on broth medium containing 0.18% agar (Duckworth & Turvey, 1969). The culture medium was harvested by centrifugation at 4000×***g***. and transferred to 3 °C. Cold, solid ammonium sulphate was slowly stirred in to a concentration of 70% (w/v). The precipitate was allowed to settle overnight, and was collected by centrifugation at 40,000×***g***. The pellet was redissolved in 0.1 M sodium phosphate buffer (pH=7.0) containing 0.1% bovine serum albumin. This was dialyzed to obtain the crude agarase which was then freeze dried and stored at −15 °C. The agarase was dissolved in sterile seawater for preparing protoplasts.

Enzymes from the abalone (*Haliotis kamtschatkana*) gut system were extracted at three different times (Chen & McCracken, 1993): Abalone ABa (enzyme stored at −20 °C for >1 year) Abalone ABb (enzyme stored at −20 °C for 1 year), and Abalone ABc (enzyme stored 1 month). The abalone enzyme mixture was dissolved in 0.4 M mannitol in seawater. After protoplasts were released, the mannitol concentration was gradually reduced to zero (Chen *et al.*, 1988).

Three commercially prepared enzymes commonly used for protoplast isolation from marine algae, Cellulase 'Onozuka' R-10, Cellulysin (Calbiochem) and Macerozyme (Yakult Honsha Ltd.), were also evaluated.

Substrate preparation and determination of enzyme activity

The protein content of the various enzyme preparations was determined using Coomassie Blue (McKnight, 1977). The substrates tested were microcrystalline cellulose (Avicel), water-insoluble *Porphyra* cell wall material prepared from *P. linearis* in 40.3% dry weight yield, (for procedure see Mukai & Craigie, 1981), xylan from oat spelts (Sigma), pophyran extracted from *P. linearis* (for procedure see Mukai & Craigie, 1981) and agarose (Sigma). Cellulase and xylanase activities were assayed in 50 mM sodium acetate buffer, pH 4.8 at 50 °C; agarase activity was measured in acetate buffer at 40 °C and pH 6.0. Enzyme activity was assayed as an increase in reducing power (Nelson, 1944).

Protoplast isolation

Enzyme mixtures of various concentrations and composition were prepared and tested for use in protoplast isolation. The yield and viability of protoplasts was determined by microscopic examination of ten aliquots of isolated protoplasts which were randomly selected from each of the agarase and abalone treated samples and stained with 0.5% Evans Blue (Sigma) in seawater.

Protoplast regeneration

After isolation, purification and resuspension in D-11 medium, protoplasts were kept in disposable petri dishes and cultured under the following conditions: 15 °C, 3:3 h (light:dark); 10 °C, 12:12 h (L:D); 10 °C, 16:8 h; 5 °C, 12:12 h (L:D); and 5 °C, 8:16 h, under a photon flux density of 10–20 μmol m^{-2} s^{-1}. During the first week, the medium containing antibiotics (Chen *et al.*, 1988) was changed daily. After one week, the antibiotic treatment was discontinued and D-11 medium was changed every 2–3 days.

Results

Substrate specificities of five enzyme preparations were determined as shown in Table 1. Protein content of the preparations as percent of dry weight were: Abalone ABa=11.0%, abalone ABb=35.5%, abalone ABc=11.0%, Cellulase R-10=9.7%, Cellulysin=10.1%, Macerozyme=1.57% and agarase=9.3%.

The most recently extracted abalone enzyme (ABc) showed greater specific activity against porphyran than the older abalone enzyme mixture (Table 1). Macerozyme showed significant activity against agarose and porphyran and also had greater cellulose-decomposing ability than Cellulase R-10. As was expected, agarase showed the greatest specific activity against porphyran and agarose.

Table 1. Substrate specifities of various enzymes. Enzyme activity is expressed as 1 unit = 1 μg glucose mg^{-1} protein min^{-1}. Reaction conditions are given in the text.

Substrate	Enzyme				
	ABb*	ABc**	Cell. R-10	Macerozyme	Agarase
Cellulose	0	0	9.0	32.7	–***
Porphyran	0.2	1.1	2.7	21.7	35.7
Agarose	–	1.9	–	41.7	622.7
Xylan	0	0	0	0	0
Cell wall material	0	0	8.1	112.5	0

* One-year-old extract. ** One-month-old extract. *** Not tested.

Few viable protoplasts were obtained when a solution containing 0.2% aged abalone (ABa or ABb) enzyme mixture was used in combination with 1% Macerozyme and up to 3% Cellulase R-10. Protoplasts were released when fresh abalone (ABc) enzyme mixture was used in combination with Macerozyme and/or Cellulase R-10, but not by the abalone enzymes alone. A range of concentrations of abalone (ABc) enzyme from 0.1% to 3.0% in combination with Macerozyme and Cellulase R-10 was tested in a series of experiments (data not shown). The optimal concentration of abalone enzyme (ABc) for isolating protoplasts from *P. linearis* fronds was found to be 0.2%. Healthy protoplasts were obtained after 2 h of enzymatic digestion using a concentration as low as 0.001% (w/v) agarase, while 0.10% agarase yielded large quantities of healthy protoplasts after only 10–15 min. Even after 4 h at a concentration of 0.1% or 24 h at 0.05%, the agarase seemed to have no effect on protoplast viability, as indicated by the lack of green debris and released phycoerythrin. This contrasts with protoplast isolations using abalone enzyme mixtures where protoplast mortality was indicated by phycoerythrin release and the production of green-coloured debris (Table 2).

Our studies on the effects of enzyme concentration on degradation of *Porphyra* tissue showed that almost all plant tissue was digested at concentrations of 0.025% to 0.10% agarase in seawater after 4 h. We selected 0.025% agarase as the most suitable concentration for isolating protoplasts from leafy thalli of *P. linearis* (Table 2).

The mean percentage of viable protoplasts obtained using the agarase enzyme process was more than 97%, while only 85% viability was observed with the abalone enzyme mixture. Microscopic observation

Table 2. Number of protoplasts ($10^6 \cdot mg^{-1}$ fresh wt of thallus) released using various concentrations of agarase (dry wt%) and abalone (0.2% w/v) enzyme mixture (A), Macerozyme (0.5%) (M) and Cellulase R-10 (2%) (C).

Enzyme	Time (h)		
	2	4	24
Agarase concentration			
0.001%	4.3	7.3	–*
0.005%	9.0	–	–
0.010%	11.5	24.2	–
0.025%	13.8	28.3	–
0.05%	18.9	28.4	26.5
0.10%	23.0	28.6	–
Abalone mixture			
A+M	7.2	15.3	–
A+M+C	12.0	21.2	N/A**

* Not tested

** Released protoplasts became green

indicated that protoplasts carried through the purification procedure maintained their pinkish-red pigmentation and normally-shaped chloroplasts. During the early stages of protoplast isolation, a number of irregularly-shaped protoplasts were observed. After 24 h these protoplasts were no longer seen and all appeared spherical.

After 6 days, protoplasts regenerated cell walls and cell division was observed under all conditions. Many cells elongated and produced one or more bud-like projections which, after 10 days, developed into conchocelis filaments and blades, particularly at 15 °C, 3:3

h (L:D); 10 °C, 12:12 h (L:D) and also at 10 °C, 16:8 h (L:D) where more than 70% of protoplasts developed into conchocelis. At 5 °C with photoperiods of 12:12 h (L:D) and 8:16 h (L:D), most protoplasts maintained their round shape, and two to three weeks later 50–60% of the protoplasts slowly developed into callus-like cell colonies, while a few developed into new leafy plantlets and conchocelis. At 10 °C and 15 °C, no callus-like cell aggregations were observed, and blade-like plantlets began forming earlier than when cultured at 5 °C. Protoplasts showed the same developmental tendencies whether isolated with abalone enzymes or with agarase. A photoperiod of 3:3 h (L:D) and higher temperature accelerated the cell differentiation.

Discussion

Partially purified agarase prepared from *Pseudomonas atlantica* used in this study is known to be a mixture of β-agarase I and β-agarase II (Morrice *et al.*, 1983). The preparation showed very strong activity against agarose and porphyran and was successfully used in protoplast isolation. Agarase was superior to the complex enzyme mixture for use in protoplast isolation because it caused less mortality of newly released protoplasts. A relatively small amount of agarase dissolved in seawater alone without added osmoticants, which have been reported to create a hypertonic solution necessary for plasmolysis (Cocking & Evans, 1974), can be used to effect the release of large numbers of viable protoplasts from *P. linearis* tissue. Another advantage of using the agarase enzyme is its long shelf life as we used agarase stored for ten years at −15 °C. Obtaining crude agarase from *Pseudomonas atlantica* is relatively simple so a ready supply of enzyme of high activity and consistent properties can be produced for commercial use. A significant additional advantage of using crude agarase in a commercial operation is that careful timing of tissue digestion becomes unnecessary as the protoplasts remain stable for at least 24 h in the presence of the enzyme. It is unnecessary to add organic osmoregulators thereby simplifying the isolation medium. As well as shortening the time for obtaining the protoplasts, ommission of the osmoregulators also reduces the carbon sources available for contaminant bacteria. The presence of dead and moribund protoplasts in the abalone-Macerozyme-Cellulase R-10 cocktail contrasts with the healthy protoplasts generated with agarase. Additional enzymes such as proteases, lipases and oxidases may be present in abalone which irreversibility damage protoplast membranes so that by 24 h no viable protoplasts can be recovered (Table 2).

The duration of storage of the extracted abalone enzymes affected its activity against porphyran. The difference in protein content and enzymatic activity expressed between crude enzymes extracted at different times also reflect variations in the physiological condition of the abalone. The newly extracted abalone enzyme had stronger activity against porphyran than the older abalone enzyme mixtures. This inconsistency of the abalone enzymes makes their performance in protoplast isolation from *P. linearis* difficult to predict and repeat.

The release of conchospores from *P. linearis* conchocelis is unpredictable at the present time. Agarase, however, can be used under standardized conditions to obtain large numbers of protoplasts. Therefore, protoplasts may be generated as a substitute for conchospores to seed nets for commercial aquaculture of *P. linearis*.

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