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ORIGINAL ARTICLE

Protoplast Preparation from *Laminaria japonica* with Recombinant Alginate Lyase and Cellulase

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Abstract Functional recombinant abalone alginate lyase (rHdAly) and β-1,4-endoglucanase (rHdEG66) were expressed as secreted proteins with baculoviral expression systems. The specific activity of each recombinant enzyme, 2,490 and 18.2 U/mg for rHdAly and rHdEG66, respectively, was comparable to its native form at 30°C. Purified rHdAly and rHdEG66 showed the highest specific activity both at 35°C and optimum pH 8.7 and 5.9, respectively. These properties were also comparable to those of the native enzymes. Protoplast isolation was attempted from *Laminaria japonica* using both rHdAly and rHdEG66. When *L. japonica* blades were incubated in artificial seawater containing rHdAly and rHdEG66, very low numbers of protoplasts ($<1\times10^3$ protoplasts/g fresh weight) resulted. However, using blades pretreated with proteinase K, the protoplast was increased up to 5×10^6 protoplasts/g fresh weight. Since the average diameter of isolated protoplasts was 11.6 μm, these cells were mostly derived from the epidermal layer rather than the cortical layer. Our results suggest that at least three enzymes, alginate lyase, cellulase, and protease, are essential for effective protoplast isolation from *L. japonica*. The protoplast isolation method in this study is more useful than earlier methods because it preferentially yielded protoplasts of the epidermal layer, which are known to be able to be regenerated.

Keywords Alginate lyase · Cellulase · Abalone · Brown algae · *Laminaria japonica* · Protoplast

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Introduction

The protoplast, which lacks a cell wall and polysaccharide sheath, is a useful tool for plant research. A method to isolate protoplasts from the brown alga *Laminaria* sp. was first reported in 1984 (Saga and Sakai 1984), followed by other studies on improved isolation methods of protoplasts from brown algae (Fisher and Gibor 1987; Polnefuller and Gibor 1987; Ducreux and Kloareg 1988, Butler et al. 1989; Boyen et al. 1990; Sawabe et al. 1993; Wakabayashi et al. 1999; Matsumura et al. 2000; Inoue et al. 2008). Since the cell wall of *Laminaria* sp. contains abundant cellulose and alginate, a combination of cellulase and alginate lyase is commonly utilized to degrade and remove cell wall components to form protoplasts. Cellulase is commercially available and prepared from fungi such as *Trichoderma* sp.; it hydrolyzes the β-1,4 glycosidic bonds of cellulose, which comprises D-glucose units (Coughlan 1985; Wood 1989). Alginate lyase, which catalyzes the degradation of alginate, is an unbranched linear heteropolysaccharide consisting of α-L-gluronate and β-D-mannuronate, by a β-elimination mechanism; it has been purified not only from bacteria (Wong et al. 2000) but also from marine invertebrates (Muramatsu et al. 1977; Boyen et al. 1990, Muramatsu et al. 1996; Shimizu et al. 2003; Suzuki et al. 2006).

Utilization of crude or partially purified enzymes for protoplast isolation raises the potential of contaminating enzymes, e.g., protease, lipase, nuclease, and carbohydrolase, causing damage to the protoplasts. *Laminaria* protoplasts can be isolated at high yield (10^6–10^7 protoplasts/g fresh weight), but their regenerability is reportedly very low, and abnormal growth of protoplasts is often observed (Sawabe and Ezura 1996; Matsumura et al. 2000). Regardless of whether the low regeneration is caused only by contaminating enzymes, the best way to overcome this problem is to use purified enzymes

for protoplast isolation. Purified alginate lyase from *Alteromonas* sp. strain H-4 (Sawabe and Ezura 1996) or from abalone *Haliotis discus hannai* in combination with a commercial cellulase was effective for protoplast isolation from *Laminaria japonica* (Inoue et al. 2008). In addition, we reported protoplast isolation using purified recombinant HdAly expressed in the cytosol of insect (Inoue et al. 2008) or yeast (Yamamoto et al. 2008) cells. In contrast, a commercial cellulase, which contains other enzyme activities in addition to β-1,4-glucanase activity, was used in almost all these studies on protoplast isolation from *Laminaria* sp., and the utilization of purified cellulase has still not been reported.

Abalone feed on a variety of algae including *Laminaria* sp. (Day and Fleming 1992; Shepherd and Steinberg 1992), and enzymes in their digestive fluid are responsible for breaking down structural polysaccharides. Thus, we isolated and characterized two alginate lyases, endolytic HdAly (Shimizu et al. 2003) and exolytic HdAlex (Suzuki et al. 2006), in addition to β-1,4-endoglucanase HdEG66 (Suzuki et al. 2003) from the digestive fluid of *H. discus hannai*, followed by cloning of their corresponding cDNA. As these enzymes are known to be involved in algal polysaccharide degradation in vivo, they should be efficacious for protoplast isolation from *Laminaria* sp.

In this paper, functional recombinant HdAly (rHdAly) and recombinant HdEG66 (rHdEG66) were expressed as secreted proteins in insect cells using a baculoviral expression system, and their enzymatic properties were subsequently characterized. Their efficacy for protoplast isolation from *L. japonica* was also investigated.

Materials and Methods

Materials

L. japonica (60–80 cm) fronds were harvested at Nanaehama near Hakodate in Hokkaido, Japan, in May 2007. DNA restriction and modification enzymes were purchased from Takara (Shiga, Japan) unless otherwise described. Native HdAly was prepared from *H. discuss hannai* (Shimizu et al. 2003). Sodium alginate from *Macrocystis pyrifera* (Sigma-Aldrich, St. Louis, MO), carboxymethylcellulose (CMC) (Kanto Chemical, Tokyo, Japan), and casein (Wako Pure Chemical Industries, Tokyo, Japan) were used as substrates for alginate lyase, cellulase, and protease, respectively.

Construction of Baculoviral Expression Systems for rHdAly and rHdEG66

Nucleotide sequence encoding honeybee mellitin signal (MKFLVNVALVFMVVYISYIA) (Habermann and Jentsch 1967; Vlasak et al. 1983) was inserted ahead of *Bam*H I site of the pFastBac1 vector (Invitrogen Corp., Carlsbad, CA) by polymerase chain reaction (PCR)-based mutagenesis. Then, nucleotide sequences encoding c-myc (EQKLI SEEDL) (Evan et al. 1985) and linker (AGGTKSG) peptides followed by an 8xHis-tag (HHHHHHHH) were inserted in this order behind *Xho* I site of the pFastBac1 vector with the same method. *Bam*H I and *Xho* I restriction sites were introduced at the 5′- and 3′-terminal ends of each cDNA encoding the mature form of HdAly (residues 18–273) or HdEG66 (residues 15–594) by PCR. After sequence confirmation using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA), each cDNA was subcloned between the *Bam*H I and *Xho* I sites in the above-mentioned modified pFastBac1 vector. Recombinant viruses were prepared according to the manufacturer's protocol. The resultant protein encodes the honeybee mellitin signal, mature form of HdAly or HdEG66, c-myc epitope sequence, linker, and 8xHis-tag in this order.

Expression, Secretion, and Purification of rHdAly and rHdEG66

Sf9 cells were infected with recombinant virus at a multiplicity of infection of 10 and then cultured in 100 ml serum-free medium (Sf900II SFM, Invitrogen) at 17°C for 72 h using a 250-ml spinner flask. After centrifugation at 2,500×*g* for 15 min, the supernatant was subjected to ammonium sulfate precipitation (40–100% for rHdAly and 20–60% for rHdEG66). The precipitate forming after centrifugation at 10,000×*g* for 15 min was dissolved in buffer containing 20 mM KPi (pH 8.0), 10 mM imidazole, and 0.5 M NaCl and then applied to Ni-NTA (Qiagen, Hilden, Germany) column (1.2×2 cm) chromatography. The resin was washed with 30 mM imidazole-HCl (pH 8.0) and 0.5 M NaCl, and protein was eluted with 150 mM imidazole-HCl (pH 8.0) and 0.5 M NaCl. Fractions containing rHdAly or rHdEG66 were dialyzed against 10 mM KPi (pH 8.0) and 0.5 M NaCl and then stored on ice until use. Protein concentration of the fractions was determined by the Bradford method (Bradford 1976). Recombinant enzymes were assayed and used within 3 days.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Porzio and Pearson (1977). A Protein Marker Broad Range (New England Biolabs, Beverly, MA) was used as a molecular mass standard in the gel.

Measurement of Enzyme Activities

The alginate lyase activity of rHdAly was determined by measuring the increase in absorbance at 235 nm using a spectrophotometer (Hitachi High-Technologies, Hitachi, Japan). One unit of lyase activity was defined as the amount of enzyme that increased the absorbance at 235 nm by 0.01 unit after 1 min.

The cellulase activity of rHdEG66 was determined by measuring the amount of reducing sugar liberated by hydrolysis of CMC using the method of Park and Johnson (1949). One unit of cellulase activity was defined as the amount of enzyme that liberated reducing sugars equivalent to 1.0 μmol glucose after 1 min.

The protease activity of recombinant proteinase K (Roche Applied Science, Penzberg, Germany) or Cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan) was measured under 10 mM KPi (pH 7.0) condition at 30°C, in which 0.1 mg/ml alkali-denatured casein was used as the substrate. The reaction was terminated by addition of the same amount of 0.11 M trichloroacetic acid. After centrifugation at 10,000×*g* for 15 min, the absorbance of the supernatant was monitored at 280 nm. One unit of protease activity was defined as the amount of enzyme that increased the absorbance at 280 nm by 0.01 unit after 1 min.

Protoplast Preparation from *L. japonica*

Protoplast preparation from *L. japonica* was performed as described previously (Inoue et al. 2008) with minor modifications. Blades of *L. japonica* fronds were chopped into small pieces and incubated in artificial seawater (Sealife, Marinetech, Tokyo, Japan) containing 0.6 M mannitol with or without 0.02 mg/ml recombinant proteinase K (Roche) at 17°C on a rotary shaker (30 rpm). The chopped blades were incubated in artificial seawater containing 0.6 M mannitol, cellulase (1% Cellulase Onozuka R-10 or various concentrations of rHdEG66), and 100 U/ml rHdAly at 17°C for 3 h on a rotary shaker (30 rpm). Next, ethylenediamine tetraacetic acid (EDTA) was added at 10 mM, and the blades were incubated for an additional hour under the same conditions. Finally, protoplasts were collected by centrifugation at 500×*g* for 5 min. The number of protoplasts was determined with a hemocytometer under a microscope. Cells were also observed under UV light in the presence of 0.01% Calcofluor white (fluorescent brightener 28, Sigma), which is a specific dye for β-glucans. Protoplast viability was determined by the method of Saga (1989) using neutral red. Stained protoplast count was done from 100 protoplasts isolated in each three experiments.

Results

Baculoviral Expression and Purification of rHdAly and rHdEG66

The recombinant enzymes rHdAly and rHdEG66 were expressed as C-terminal His-tag fusion proteins (Fig. 1a). Replacing the native signal peptide sequence with the honeybee mellitin signal allowed secretion of the recombinant enzymes. There was no secretion when expression of the enzymes was attempted with the native signal peptide (data not shown), indicating that the abalone native signal peptide was unable to function properly in insect cells. At first, recombinant enzyme could be purified from the medium after culturing at 28°C for 72 h, but the specific activities of rHdAly and rHdEG66 were only 15% and 3%, respectively, compared to their native forms (data not shown). Since expressed proteins were considered to be denatured during incubation at this temperature, we examined the production of protein at various culture temperatures (4°C, 10°C, 15°C, 17°C, 20°C, or 23°C) (data not shown); the highest activities of both rHdAly and rHdEG66 were achieved at 17°C. Therefore, incubation temperature for expression of recombinant enzymes was set at 17°C. Each purified enzyme was detected on SDS-PAGE as a single band with the predicted molecular weight (Fig. 1b). We obtained 1.6 mg rHdAly and 4.8 mg rHdEG66 from a 1-l cell culture.

Characterization of rHdAly and rHdEG66

We examined the properties of the purified rHdAly and rHdEG66. The specific activity of rHdAly was 2,490 U/mg at 30°C in buffer containing 10 mM NaPi (pH 8.0). The optimum temperature and pH were 35°C and 8.7, respectively (Fig. 2a, b). The activity of rHdAly was decreased by 50% after incubation at 50°C for 30 min (Fig. 2c).

rHdEG66 showed an activity of 18.2 U/mg for the decomposition of CMC at 30°C in buffer containing 10 mM NaPi (pH 8.0) but did not degrade crystalline cellulose such as Avicel under the same conditions, even after 48 h. The optimum temperature and pH were 35°C and 5.9 using CMC as the substrate. The CMCase activity of rHdEG66 was decreased by 50% after incubation at 50°C for 30 min.

Thus, we succeeded in expressing recombinant abalone alginate lyase and β-1,4-endoglucanase as functional proteins.

Protoplast Preparation from *L. japonica* Using rHdAly and rHdEG66

Isolation of *L. japonica* protoplasts using 100 U/ml rHdAly and 1% Cellulase Onozuka R-10 resulted in 1×10^7 protoplasts/g fresh weight (Fig. 3); this protoplast yield

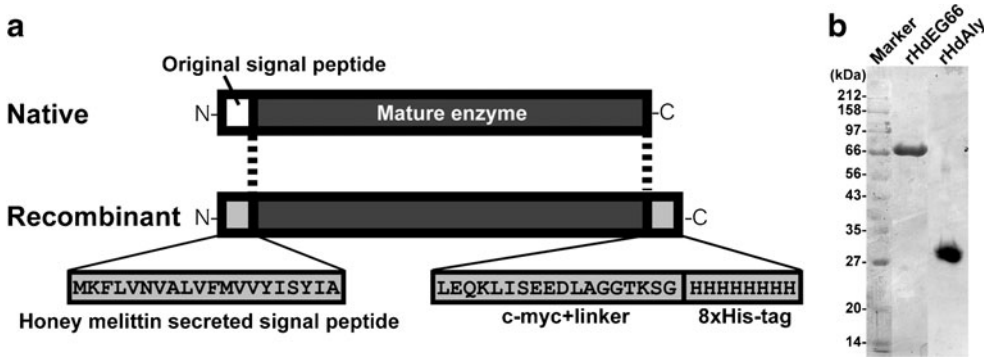

Fig. 1 Baculoviral expression of rHdAly and rHdEG66. **a** Schematic drawing of native and recombinant enzymes. **b** SDS-PAGE patterns of purified rHdAly and rHdEG66. Sizes of molecular markers are shown on the *left*

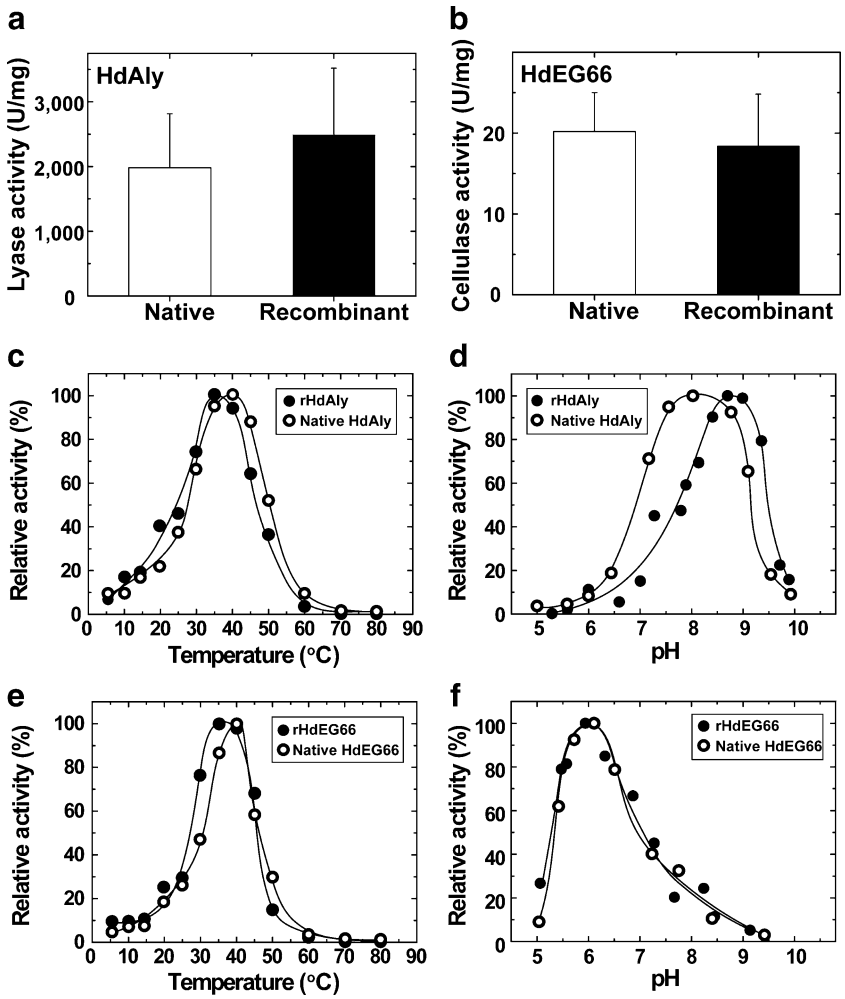

Fig. 2 Fundamental properties of recombinant enzymes. Enzyme activities of HdAly (**a**, **c**, **e**) or HdEG66 (**b**, **d**, **f**) were measured at 30° C unless otherwise mentioned here. **a**, **b** Comparison of the activities of native and recombinant enzymes. The activities of HdAly and HdEG66 were measured at pH 8.0 and 5.5, respectively. *Empty* and *filled bars*, respectively, show the activity of native and recombinant enzymes. *Error bars* show SD of three independent samples. **c**, **e** Temperature-dependent enzymatic activities. Each activity was measured under the same conditions as in **a** and **b** at the indicated temperatures. For native HdAly and rHdAly, 100% activity corresponds to 2,360 and 2,820 U/mg, respectively (**c**). For native HdEG66 and rHdEG66, 100% activity corresponds to 20.2 and 21.8 U/mg, respectively (**e**). **d**, **f** pH-dependent enzymatic activities. Each activity was measured in 10 mM NaPi with the pH adjusted as indicated at 30° C. For native HdAly and rHdAly, 100% activity corresponds to 2,610 and 3,220 U/mg, respectively (**d**). For native HdEG66 and rHdEG66, 100% activity corresponds to 19.4 and 22.3 U/mg, respectively (**f**). *Filled* and *open circles* show recombinant and native enzymes, respectively (**c**–**f**)

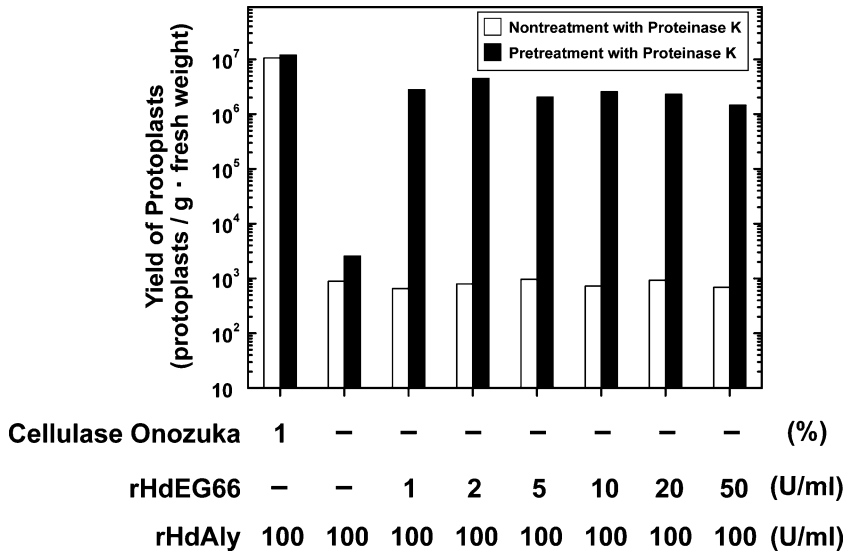

Fig. 3 Effects of various enzymes on protoplast isolation from *L. japonica*. *Empty* and *filled bars*, respectively, represent yields of protoplasts using untreated blades and blades pretreated with proteinase K. The amount and type of added enzyme are shown *under the panel*

was comparable to that obtained using the same activities of native HdAly and Cellulase Onozuka. On the other hand, substitution of rHdEG66 for Cellulase Onozuka markedly reduced the protoplast yield to 1×10^3 protoplasts/g fresh weight, which was almost the same yield as that using rHdAly without cellulase (Fig. 3). These results support that recombinant HdAly has the capacity to degrade alginate components for protoplast isolation as reported previously (Inoue et al. 2008; Yamamoto et al. 2008), but rHdEG66 was insufficient for preparing protoplasts due to inefficient degradation of cellulose components.

Cellulase Onozuka is not a highly purified enzyme; according to the manufacturer's manual, it contains other enzyme activities including cellulase activity. Here, we focused on protease activity in Cellulase Onozuka because structural proteins exist in the cell wall. Measurement of protease activity using casein as the substrate revealed that Cellulase Onozuka had a specific activity of 1.53 U/mg at 30°C and pH 7.0. This result indicates that the protoplast isolation solution contained 15.3 U of protease activity, although it is uncertain what kind of protease is active in Cellulase Onozuka. Next, we examined the effect of protein digestion on protoplast isolation from *L. japonica* using commercially available recombinant purified proteinase K. For protoplast manipulation, 0.02 mg/ml proteinase K, which showed an activity of 22.6 U/ml at 30°C, was added to a hypertonic solution containing 100 U/ml rHdAly and 5 U/ml rHdEG66, resulting in no protoplast formation even after 12 h incubation with EDTA (data not shown). We postulated that rHdAly and rHdEG66 were digested by proteinase K before cell wall polysaccharide degradation. Thallus pieces were thus preincubated in a hypertonic solution containing only 0.02 mg/ml proteinase K for 15 min at 17°C and then washed with ten volumes of the same solution without proteinase K for a following incubation with rHdAly and rHdEG66. This method allowed protoplast isolation using rHdAly and rHdEG66. As shown in Fig. 3, protoplast yields were significantly elevated by the addition of 1 U/ml rHdEG66 and reached a maximum of 5×10^6 protoplasts/g fresh weight when proteinase K-pretreated thallus was treated with 100 U/ml rHdAly and 2 U/ml rHdEG66. These results suggest that the combined use of these three enzymes, rHdAly, rHdEG66, and proteinase K, is effective for protoplast isolation from *L. japonica*.

Protoplast size depended on the kind of cellulase (Fig. 4). The average diameter of protoplasts prepared by rHdEG66 was 11.6 μm, which was about 40% the size of protoplasts prepared by Cellulase Onozuka. Thus, protoplasts were predominantly isolated from the epidermal layer of the thallus when rHdEG66 was used instead of Cellulase Onozuka. Calcofluor staining revealed that the freshly isolated protoplasts using rHdAly and rHdEG66 did not have cell wall polysaccharides (Fig. 4d). Although some cellular masses were also observed at the same condition, these cells contained cell wall (Fig. 4d, inset). The viability of prepared protoplasts using rHdAly and rHdEG66 was assessed with neutral red staining as shown in Fig. 4e and determined as 93.8±2.8% (mean ± SD). This viability was comparable to that of protoplasts isolated from *L. japonica* with marine bacterial alginate lyase and commercially available cellulase (Sawabe and Ezura 1996).

Discussion

Abalone is a marine invertebrate that feeds on macroalgae including brown algae, but its digestive mechanism

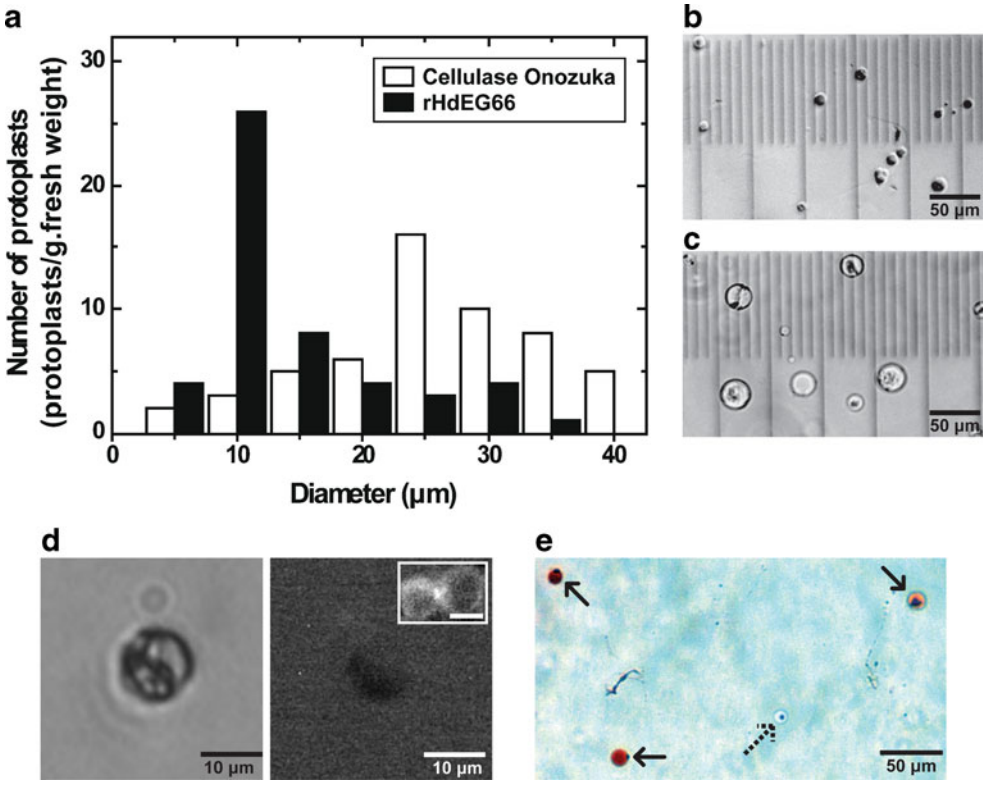

Fig. 4 Sizes of protoplasts isolated using recombinant enzymes. **a** Protoplast yield with an enzyme mixture of 100 U/ml rHdAly and 1% Cellulase Onozuka (*empty bars*) or 5 U/ml rHdEG66 (*filled bars*). Blades were pretreated with 0.02 mg/ml proteinase K under both conditions. **b**, **c** Protoplasts isolated with a mixture of rHdAly and rHdEG66 (**b**) or with Cellulase Onozuka (**c**). A freshly isolated protoplast from *L. japonica* using rHdAly and rHdEG66 in bright field (**d**, *left*) and under UV light in the presence of Calcofluor (**d**, *right*). *Inset* (**d**, *right*) shows Calcofluor-stained cellular mass observed during protoplast preparation at the same condition. Neutral red-stained protoplasts from *L. japonica* using rHdAly and rHdEG66 (**e**). The *solid arrows* and the *dashed arrow* indicate stained cells and an unstained cell, respectively. *Solid bars* show 50 μm (**b**, **c**, **e**) and 10 μm (**d**)

remains unclear. We have isolated and characterized HdAly (Shimizu et al. 2003) and HdEG66 (Suzuki et al. 2003) from *H. discus hannai*, enzymes in abalone digestive fluid that are thought to be involved in degradation of the polysaccharide components of their food. This idea suggested the possibility of protoplast isolation from *L. japonica* through the decomposition of cell wall by HdAly and HdEG66 because early studies succeeded in high yield protoplast isolation from *Laminaria* sp. by utilizing alginate lyase and cellulase. We have shown that a native or recombinant HdAly mixture with Cellulase Onozuka is suitable for protoplast isolation (Inoue et al. 2008; Yamamoto et al. 2008). In our previous studies, although recombinant HdAly could be expressed in the cytosol of insect cells or yeast, a large fraction of the expressed protein precipitated. In this study, we attempted cytosolic expression of HdEG66 in insect cells; however, functional HdEG66 could not be expressed or purified, probably due to an abnormal structural conformation and aggregation in the cytosol of insect cells (data not shown). Since these enzymes are naturally secretion proteins, their expression should be achieved through the secretion pathway. In fact, the utilization of a secretory expression system allowed sufficient yield of rHdAly and rHdEG66, as expected. In this study, the protein expression temperature was set at 17°C, which was about 10°C lower than the generally used temperature for protein expression in insect cells. Both rHdAly- and rHdEG66-obtained cultures at ≥20°C showed a significant reduction in activity. These observations are presumably related to the preference of abalone at low temperatures. Protein expression at ≤15°C caused a remarkable decrease in the amount of recombinant enzyme, probably due to a decrease in insect cell activity

Effective protoplast isolation from *L. japonica* requires a combination of at least three enzymes, alginate lyase, cellulase, and protease (Fig. 3). To date, research on protoplast isolation from *Laminaria* sp. has largely focused on polysaccharide degradation and related enzymes. Treatment of thallus by two purified enzymes, rHdAly and rHdEG66, alone was insufficient for degradation of cell wall components, with protoplast yield under these conditions being almost equal to that using only rHdAly (Fig. 3). This observation indicates that rHdEG66 cannot

significantly degrade cellulose components in the *L. japonica* cell wall under these conditions. In contrast, in the absence of protease, cell wall proteins are expected to maintain the cell wall structure even after degradation of cellulose by rHdEG66. Although there is no information on cell wall proteins in *Laminaria* sp., structural proteins similar to those in other plants are predicted to function in laminarian cell walls. In higher plants, polysaccharide cell wall components bind glycoproteins, which are usually rich in proline, hydroxyproline, and lysine, through hydrogen and/or electrostatic bonds (Lamport 1965; Cassab 1998). Several hydroxyproline-rich glycoproteins having common hydrophilic and hydrophobic motifs in plant cell walls comprise the extensin family, which bind not only with each other but also with cellulose fibers, resulting in cell wall assembly (Fry 1982; Epstein and Lamport 1984; Kieliszewski and Lamport 1994; Cassab 1998). If proteins such as extensin were in the cell wall of *Laminaria* sp., degradation of only cellulose by cellulase would still be insufficient to break down the rigid cell wall structure, and thus protein digestion would also be required. In the case of alginate, degradation by alginate lyase and decomposition of its gel structure through the removal of divalent metal ions by EDTA were critical for isolation of protoplasts from *L. Japonica* (Inoue et al. 2008).

The epidermal layer likely yielded protoplasts preferentially when using purified rHdAly, rHdEG66, and proteinase K (Fig. 4). Protoplasts isolated using commercial cellulase were from the cortical layer rather than the epidermal layer. Only cells derived from the epidermal layer were reported to be regenerable (Matsumura et al. 2000). Therefore, the method in this study is more suitable than earlier methods for obtaining more vigorous protoplasts.

The difference in tissue origin of protoplasts observed in this and other studies can be explained by the β-1,4-glucanase properties of fungal cellulase and abalone HdEG66. *Trichoderma* sp. produces three kinds of cellulases; e.g., *Trichoderma reesei* secretes at least five β-1,4-endoglucanases, two cellobiohydrolases (β-1,4-exoglucanases), and two β-glucosidases (Kubicek 1992; Saloheimo et al. 1994; Nevalainen and Pentilla 1995; Lynd et al. 2002). Of these, β-1,4-endoglucanases and cellobiohydrolases are likely to be involved in cell wall degradation, while β-glucosidases preferentially hydrolyze cellobiose and cellooligosaccharides resulting from cellulose digested by β-1,4-endoglucanase and cellobiohydrolase. Moreover, cellobiohydrolase works well on crystalline cellulose, releasing cellobiose, while β-1,4-glucanase mostly attacks noncrystalline or amorphous cellulose. Such substrate specificity would likely be closely related to the degradable portion of algal cell wall and the size of isolated protoplasts from *L. japonica*. Although the detailed structure of cellulose in *Laminaria* sp. is not yet clear, the crystallinity of cellulose likely differs between epidermal and cortical cell walls. In addition, since inner cortical cells are older than outer epidermal cells, the content of crystalline cellulose in the cortical layer would be higher than that in the epidermal layer. For this reason, β-1,4-endoglucanase such as HdEG66 can selectively attack cellulose of epidermal cells, while Cellulase Onozuka, containing cellobiohydrolase and β-1,4-endoglucanase, can act on cellulose of both epidermal and cortical cells. Thus, the difference in the average size of protoplasts can be attributed to the substrate specificity of the cellulase used.

In conclusion, this study revealed that at least three enzymes, alginate lyase, cellulase, and protease, are essential for effective protoplast isolation from *L. japonica*. An insect cell-secreted protein expression system was appropriate for both HdAly and HdEG66. Furthermore, substrate specificity of cellulase was a key for determining the origin of protoplasts. Culture and regeneration studies of protoplasts obtained using purified recombinant enzymes are under way.

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