Selection of a L-Lysine-Overproducing Strain of the Red Seaweed *Porphyra suborbiculata* (Rhodophyta) through Mutation and Analog Enrichment

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Abstract

An improved strain of the red seaweed *Porphyra suborbiculata* containing an increased amount of the essential amino acid L-lysine was obtained through mutation and analog enrichment. Mutagenesis using a 10% lethal dose of ultraviolet irradiation and an enrichment culture with the L-lysine analog aminoethyl-L-cysteine (AEC) was repeated to select the most productive strain using monospores of *P. suborbiculata*. The concentrations of AEC required to produce 50 and 100% inhibition of survival were 60 and 115 mM in the parent strain, and 72 and 135 mM in the selected AEC-resistant strain, respectively. The AEC-resistant strain, L130, produced 1.74-fold more lysine compared to its parent strain. Thus, mutagenesis with analog enrichment shows promise for selecting seaweed strains that can overproduce this essential amino acid.

Key words: *Porphyra suborbiculata*, Enrichment culture, Lysine, Mutation, Strain improvement

Introduction

The genus *Porphyra* (Bangiales, Rhodophyta) is one of the most important edible seaweeds cultivated commercially in Korea, Japan, and China. In 2009, the Korean aquaculture industry produced 211,000 t (wet weight) from 57,000 ha (Korea Fisheries Association, 2010). This seaweed is highly prized for its flavor and as a health food rich in proteins, vitamins, and minerals. *Porphyra* also contains various biologically active substances that are beneficial to human health (Noda, 1993). Seventeen free amino acids, including taurine, which controls blood cholesterol levels, are abundant in *Porphyra* (Elvevoll et al., 2008). In addition, *Porphyra* is a source of the red pigment R-phycoerythrin, which is used as a fluorescent tag for immunofluorescence studies (Kronick, 1986).

Bilogy and ecology of *Porphyra* has been studied more thoroughly than those of any other genus of red algae (Tseng and Sun, 1989; Cole, 1990; Hawkes, 1990). Recently, *Porphyra* was reported as being suitable as an experimental system in modern biological research, similar to *Arabidopsis thaliana* (Saga and Kitade, 2002; Sahoo et al., 2002).

Until now, *Porphyra suborbiculata* was not considered to have great commercial value as a food source compared to the aquacultured strains *Porphyra yezoensis* and *Porphyra tenera*. However, *P. suborbiculata* offers a significant advantage to farmers because it can be grown from spring to summer, allowing farming in the warm-seawater season rather than just the cold-water season (Jin et al., 2000).

As has been demonstrated repeatedly in agricultural crops and other types of cultivation, the genetic improvement of a

cultured species is generally crucial to maximize yields and to obtain useful by-products. However, unlike terrestrial plants, improvement techniques for seaweed strains have generally been restricted to classic breeding methods, particularly strain selection. To date, the most successful method for producing new strains of *Porphyra* is repeated strain selection (Japanese Society of Fisheries, 1979), although the approach has many disadvantages and limitations. For example, repeated strain selection is labor-intensive and usually requires many years of rigorous effort. In addition, the existing genetic variability in one or more populations of interest may not be sufficient for strain selection. Another method for strain improvement is somatic hybridization via protoplast fusion (Fujita and Saito, 1990). Genetic engineering and mutant selection have also been suggested as appropriate methods for strain improvement (Brown et al., 1990); however, genetic engineering can be applied only to systems in which the genetic fundamentals are well developed. In contrast, mutant selection has the advantage of simplicity; it requires little knowledge of the pathways involved in the biosynthesis of the desired product and minimal technical manipulation (Rowlands, 1984). Strain improvement through mutagenesis has been broadly applied in microbial industries; e.g., the yield of penicillin was increased from 0.06 to 26 mg mL^{-1} in a stepwise fashion through the use of mutant selection (Queener and Lively, 1986).

The amino acid composition of *Porphyra* is rich with taurine, alanine, and glutamic acid, but it has low levels of lysine, an essential amino acid that is also deficient in rice (Kagawa, 1983; Noda, 1993). Thus, lysine is one of the most limiting essential amino acids in *Porphyra*-wrapped rice meals, which are popular in Korea and Japan. Lysine is the first limiting amino acid for protein synthesis in people consuming a predominantly cereal-based diet of wheat and rice (Young and Pellett, 1994). To enhance the lysine supply, we attempted to develop a lysine-overproducing strain of *P. suborbiculata*, which could also be used as an example of strain improvement in a *Porphyra* species. Although the procedure requires repeated mutagenesis and analog-enriched selection, it is simple and requires no sophisticated equipment for the selection of amino acid overproducers.

Materials and Methods

Monospore culture

Juvenile blades of *P. suborbiculata* Kjellman were collected from a rocky shore at Cheongsapo (35°09′28″ N, 129°11′47″ E), on the east coast of Busan, Korea. The fresh blades were rinsed, sonicated (60 kHz) twice for 1 min in autoclaved seawater, and immersed in 1% betadine with 2% Triton X-100 for 1 min to eliminate epiphytes. To liberate monospores, the blades were cultured in Provasoli's Enriched Seawater (PES) medium (Provasoli, 1968) under 10 h of light (40 μmol m^{-2} s^{-1})

and 14 h of dark at 20°C for 20 days. The monospores were then grown to juvenile blades. Healthy juvenile blades were selected for use as a parent strain (W1). The parent blades started to produce monospores under the same culture conditions.

Experimental design

For monospore mutation, ultraviolet (UV) radiation was used as a mutagenic agent. The UV source was a 254-nm wavelength germicidal lamp (30 W) that was placed 20 cm above the monospore-containing plates. To measure the lethal exposure time for the parent monospores, 500 μL of culture media containing approximately 1,000 monospores per well in a 24-well plate was exposed to UV light with continuous agitation; a sample was removed every 30 s. The irradiated monospores were immediately stored in darkness for 1 day to avoid photoreactivation (Carlton and Brown, 1981) and then grown to juvenile blades over a period of 1 week. Survival rate (%) of the irradiated spores compared to nonirradiated spores was calculated as the number of regenerated monospores in PES. The regenerative ability of plants is more severely affected by mutagens more so than growth potential (Moustafa et al., 1989). Thus, we selected a level of mutagenesis that did not significantly inhibit regeneration (30 s of irradiation, which yielded almost 90% survival). A sufficient number of next-generation monospores was viable at this level.

To select lysine overproducers, the UV-treated monospores were grown in PES that contained the lysine analog *S*-(2-aminoethyl)-L-cysteine (thialysine; AEC) as an enrichment culture. A total of 1 mL of PES containing ca. 1,000 monospores per well in a 24-well plate was cultured with different concentrations of AEC for 1 week, and then the regenerated monospores were counted. IC$_{50}$ (50% inhibitory concentration) and IC$_{100}$ (100% inhibitory concentration) were determined from the dose-response curve. To isolate AEC-resistant mutants in the first round, UV-treated monospores were cultured in PES containing 110 mM AEC, which was the minimum lethal concentration (minimum concentration of AEC required for 100% inhibition of cell growth; MLC) for parent monospores. After 1 week, the AEC-containing medium was exchanged with normal PES and the culture was continued until the juvenile blades released new monospores. The released monospores were used in a subsequent round of UV treatment-enrichment culture with increased concentrations of AEC for 1 week, followed by culture in normal PES. New monospores were released repeatedly from juvenile blades that survived the previous AEC concentration and were used in the following rounds until a juvenile (strain L130) showing resistance to the highest concentration of AEC could be found.

Survival and growth rates

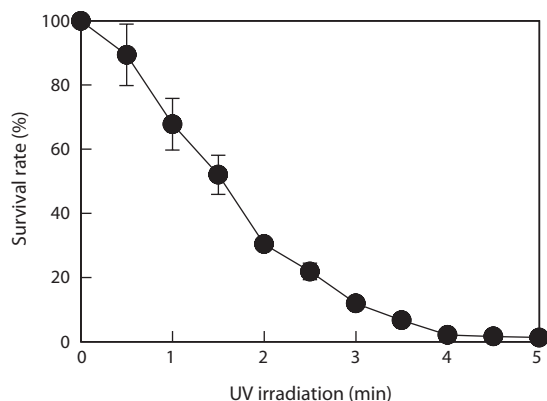Survival rate (%) of the monospores in AEC-containing

Fig. 1. Lethal time for parent monospores after UV irradiation using a germicidal lamp. A 30-W UV germicidal lamp was placed 20 cm above the monospores. The survival rate (%) of the irradiated spores against non-irradiated spores was calculated as the number of monospores regenerated in Provasoli's enriched seawater. The data are expressed as the mean ± SE ($n \geq 3$).
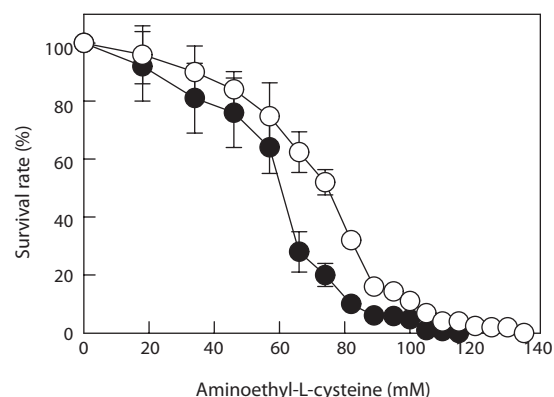

Fig. 2. Dose-response curve of *Porphyra suborbiculata* monospores with the lysine analog aminoethyl-L-cysteine. Monospores of the parent strain (closed circles) and AEC-resistant strain L130 (open circles) were cultured in Provasoli's enriched seawater (PES) containing different concentrations of AEC for one week and the regenerated monospores counted. The survival rate (%) was calculated against controls containing no AEC in PES. All data are expressed as the mean ± SE ($n \geq 3$).

PES was calculated as a relative rate: $(A/N) \times 100$, where A = the number of blades that germinated after culture in AEC-containing PES for 1 week and normal PES, and N = the number of blades after culturing in normal PES. Juvenile blades were grown in a temperature-controlled incubator under light at 40 μmol m^{-2} s^{-1} and 20°C. The cells of the blades were counted under a microscope with a hemocytometer. The specific growth rate (λ) of the blades was calculated as cell growth against culture time: $\log N - \log N_0 = \lambda(T - T_0)/2.303$, where N = the number of cells on day T and N_0 = the number of cells on day T_0.

Analysis of gross biochemical composition

Biomass dry weight was measured after washing with 0.5 M ammonium bicarbonate (pH 7.5) and drying at 95°C for 1 day (Zhu and Lee, 1997). Dried samples were ashed by heating for 5 h in an electric oven at 540°C (Association of Official Analytical Chemists, 2005). Total carbohydrate content was determined by the phenol-sulfuric acid method (Kochert, 1978) with glucose as the standard. Total lipids were extracted by hexane and isopropanol (3:2) as the solvent (Radin, 1981) and quantified gravimetrically. The amount of soluble protein in the cells was estimated by the method of Lowry et al. (1951) after heating the cell suspension at 100°C in 1 N NaOH for 2 h to achieve complete protein solubilization. Bovine serum albumin was used as the standard when determining the protein content.

Measurement of free amino acids

Dried samples (100 mg) of the blades were suspended in 5 mL of H$_2$O in a hydrolysis tube at 60°C on a heating block for 1 h. A total of 100 mg of sulfosalicylic acid was added to the homogenates, which were refrigerated (4°C) for 2 h and then centrifuged (15,000 rpm, 4°C, 15 min). The supernatant was collected and evaporated in a rotary vacuum evaporator. The residue was dissolved in 2 mL of Li-citrate buffer (pH 2.2) and filtered prior to measurement through a filter membrane (0.2 μm). Amino acids were analyzed using a Biochrom 20 amino acid analyzer (Cambridge, UK).

Results

Approximately 40 monospores (average size, 15 μm) were produced from an average juvenile blade that was 100 μm in length. Monospores and juvenile blades were separated by filtering through a 20-μm mesh nylon membrane. The conditions for mutagenesis were determined by UV irradiation over various time periods with specimens located 20 cm from a 30-W UV lamp. After irradiation, the monospore survival rate was determined (Fig. 1). Irradiation for 30 s yielded almost 90% survival. Only 10% of the monospores were destroyed by UV irradiation under these conditions; the other 90% were mutated or undamaged. All monospores died after 4 min of irradiation. Thus, we decided to use the conditions that produced a 90% survival rate (30 s of irradiation) for mutagenesis.

Resistance of the parent monospores to AEC was determined from a dose-response curve (Fig. 2). IC$_{50}$ was approximately 60 mM and IC$_{100}$ was 115 mM. Thus, the selection of AEC-resistant mutants after UV treatment commenced with a 115 mM AEC enrichment culture. After culturing the 30-s UV-irradiated monospores in 115 mM AEC for 1 week, the surviving monospores (2nd generation) were placed in fresh
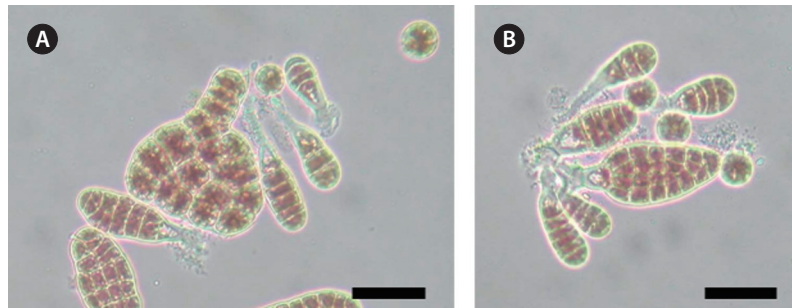
Fig. 3. Surface view of the parent strain W1 (A) and AEC-resistant strain L130 (B) of *Porphyra suborbiculata* after 20 day culture in Provasoli's enriched seawater. Scale bars: A, B = 60 μm.

PES without AEC for germination, growth to juvenile blades, and monospore production. These new monospores were mutated again by exposure to UV irradiation for 30 s and then cultured in PES containing 120 mM AEC. After 1 week of treatment, the surviving monospores (3rd generation) were placed in fresh PES to regenerate blades and monospores. These monospores were mutated in the same way and cultured in PES containing 125 mM AEC. After 1 week, the surviving monospores (4th generation) were placed in fresh PES to regenerate blades and monospores. Again, the new monospores were mutated and cultured in PES containing 130 mM AEC for 1 week. The surviving monospores (5th generation) were placed in fresh PES, and one of the rapidly growing blades was selected. The selected juvenile blade was labeled strain L130. Monospores obtained from the 5th generation were mutated and cultured in PES containing 135 mM AEC. No monospores survived at this AEC concentration. The resistance of the L130 monospores to AEC was determined from the dose-response curve (Fig. 2); IC_{50} and IC_{100} had increased to 72 and 135 mM, respectively.

The parent strain W1 and the AEC-resistant strain L130 had nearly the same spore shape (approximately 15 μm in diameter) and blade shape (Fig. 3). The specific growth rates of juvenile blades from strains W1 and L130, measured during the early growth phase between days 6 and 7, were also approximately the same at 0.42 cm per day (Table 1). In terms of gross biochemical composition, no significant differences were observed between W1 and L130 in total carbohydrate, lipid, protein, or ash content (Table 1). Free amino acid composition was also compared between the parent and AEC-resistant strains. L130 produced 174% more free lysine than strain W1 (Table 2). Strain L130 also produced 180% more phenylalanine compared to the parent strain, but it produced less leucine and serine. Alanine and taurine were dominant in strain L130 and their levels were comparable to those of the parent strain.

Table 1. Specific growth rate and gross biochemical composition of juvenile blades of the parent strain (W1) and AEC-resistant strain (L130) of *Porphyra suborbiculata**

	Parent strain W1	AEC-resistant strain L130
Specific growth rate (1/day)	0.423 ± 0.05	0.420 ± 0.02
Total carbohydrate (%)	40.5	39.7
Lipid (%)	1.3	1.3
Protein (%)	38.2	39.7
Ash (%)	14.5	14.8

*The specific growth rate (λ) was calculated as the cell number increase per culture day as the mean ± SE ($n \geq 3$). The values for gross biochemical composition are expressed on a dry weight basis (%).

Table 2. Composition of free amino acids in the parent strain (W1) and AEC-resistant strain (L130) of *Porphyra suborbiculata* on a dry weight basis

Amino acid	Amount (μg/100 mg)		Relative enhancement (%)
	Strain W1	Strain L130	
Alanine	958	962	100
Arginine	94	111	118
Aspartic acid	138	122	88
Cysteine	ND	ND	ND
Glutamic acid	327	383	117
Glycine	160	200	125
Histidine	ND	ND	ND
Isoleucine	25	26	102
Leucine	185	137	74
Lysine	38	66	174
Methionine	ND	ND	ND
Ornithine	62	65	105
Phenylalanine	40	72	180
Proline	ND	ND	ND
Serine	189	147	78
Taurine	1,550	1,360	88
Threonine	75	94	126
Tryptophan	ND	ND	ND
Tyrosine	8.7	7.7	88
Valine	50	68	136
Total	3,899.7	3,820.7	

ND, not detectable amount.

Discussion

Occurrence of mutations, even when induced by highly efficient mutagens, is relatively infrequent. For example, the mutation frequency in a bacterial population may be as low as 1 per 10^6 or 10^7 cells (Carlton and Brown, 1981). Recovering a particular mutant from such a large background of nonmutant cells can be tedious, especially when indirect detection techniques such as amino acid analysis must be employed. In such cases, an enrichment step is often required before mutant screening. The use of amino acid analogs for enrichment does not allow nonmutant cells to grow because of competitive interference with normal amino acids (Queener and Lively, 1986). The production of primary metabolites such as amino acids is regulated by metabolic feedback mechanisms (Kisumi, 1986), including feedback inhibition and repression. The destruction of feedback controls is the most important criterion for the overproduction of a desired amino acid (Kisumi, 1986). Generally, strains in which feedback controls are released can be obtained by isolating amino acid analog-resistant mutants. The lysine analogs AEC and δ-hydroxylysine inhibit the growth of wild-type *Saccharomyces cerevisiae* (Bhattacharjee, 1983). AEC-resistant mutants have been isolated from bacteria such as *Brevibacterium lactofermentum* (Tosaka et al., 1978), *Corynebacterium glutamicum* (Schrumpf et al., 1992), and *Lactobacillus plantarum* (Cahyanto et al., 2007) to increase lysine production. Aspartokinase, which is related to the feedback inhibition by L-lysine, in such mutants is desensitized; thus, the mutants become L-lysine overproducers. The regulation of L-lysine biosynthesis in *L. plantarum* suggests that amplification of genes involved in L-lysine biosynthesis will not significantly enhance L-lysine production in wild-type strains because of the inhibition of aspartokinase activity when L-lysine is in excess (Cahyanto et al., 2007). The construction of an L-lysine-overproducing strain through gene amplification, therefore, might only be feasible for a mutant strain that possesses L-lysine-insensitive aspartokinases. Most bacteria, algae, and plants use aspartate as a precursor in the α,ε-diaminopimelate pathway (Tosaka and Takinami, 1986), in which the first reaction is an ATP-dependent phosphorylation catalyzed by aspartokinase that is controlled by feedback inhibition (Garrett and Grisham, 2005).

Lysine is an indispensable amino acid that is essential in the diet of humans. However, *Porphyra* and rice, which are the major materials in *Porphyra*-wrapped rice meals, are both very low in lysine (Bhattacharjee, 1983; Noda, 1993). Although the amount of lysine produced by the *Porphyra* mutant in this study was low in comparison with production in microorganisms (Tosaka and Takinami, 1986), the overproduction of lysine or other essential amino acids in edible seaweed could significantly improve its nutritional value and marketability. Moreover, strains that produce higher amounts of other essential amino acids such as histidine, methionine, and tryptophan, which are at their lowest levels in *Porphyra*, might be obtainable by sequentially conducting a range of mutations, i.e., by repeated mutagenesis and enrichment selection against their amino acid analogs.

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