Stabilization of Astaxanthin by A Novel Biosurfactant Produced by *Rhodotorula mucilaginosa* KUGPP-1

HIDEHISA KAWAHARA*, AKIHIITO HIRAI, TOSHI MINABE, AND HITOSHI OBATA

Department of Life Science and Biotechnology, Kansai University, 3-3-35 Yamate-cho, Suita, Osaka 564-8680, Japan

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We found that a novel biosurfactant from the cultured broth of red yeast, *Rhodotorula mucilaginosa* KUGPP-1, originating in the Antarctic, has dispersive power against astaxanthin. The novel biosurfactant was purified from extracts to the ultrafiltration state by acetone precipitation and chromatography on a DEAE-Toyopearl 650M, and gel filtration on a Sephacryl S-400HR. The molecular mass of the novel biosurfactant was estimated to be about 730,000 by gel filtration chromatography. The novel biosurfactant was comprised of sugar and protein in an approximate molar ratio of 9 : 1. The sugars were comprised of mannose, galactose and glucose. The particle size of the astaxanthin (0.13 μg/ml) micelle was about 410 nm. Astaxanthin was stable to oxidation in the novel biosurfactant micelles. To our knowledge, this is the first report on a glycoprotein type of biosurfactant with astaxanthin-stabilizing ability.

Key words: Biosurfactant/Astaxanthin/Rhodotorula mucilaginosa.

INTRODUCTION

Carotenoids are liposoluble tetraterpene-type molecules which show red to yellow coloration (Britton, 1995) and can defend cells against oxygen radicals (Schoed and Johnson, 1993, 1995). The antioxidant action of carotenoids against reactive oxygen species has been well studied (Miller et al., 1996). Although this antioxidant action is expected as a functional-food material, carotenoids with a high level of anti-oxidation activity are unstable in the presence of oxygen presence at room temperature.

Carotenoids can be produced by several microorganisms, including bacteria (Bhosale and Bernstein, 2004), algae (Jeon et al., 2006), molds (Kuzina and Cerda-Olmedo, 2006), and some red yeasts (An et al., 2001, Bhosale and Gadre, 2001). The synthesis of different natural commercially important carotenoids (α-carotene, torulene, torularhodin and astaxanthin) was done using several yeast species belonging to the genera *Rhodotorula* and *Phaffia*. Among some yeast species, *Rhodotorula mucilaginosa* is the species in most frequent possession of four main pigments, that is, torularhodin, torulene, β-carotene and γ-carotene (Buzzini et al., 1998). Torularhodin, among the four carotenoids, contributes substantially to enhance UV-B tolerance in yeasts (Molline et al., 2010).

Astaxanthin has important application in the nutraceutical, cosmetics, food and feed industries. Although astaxanthin has antioxidant and anti-inflammatory abilities and provides UV-light protection, etc. (Guerin et al., 2003), carotenoids are liposoluble, water-nonsoluble molecules. Carotenoids, including astaxanthin, have been used as additives for foods and cosmetics after emulsification by proper surfactants containing chemicals and biosurfactants. Also, although astaxanthin is an unstable carotenoid, astaxanthin as a reagent for research is stored under frozen conditions.

Biosurfactants are produced by several types of microorganisms, such as bacteria, yeasts and fungi (Fiechter, 1992). Biosurfactants have attracted considerable interest due to their low toxicity,

*Corresponding author. Tel: +81-6-6368-0832, Fax: +81-6-6388-8609, E-mail:kawahara(a)pcku.kansai-u.ac.jp
biodegradable nature, and diverse structures (Banat et al., 2000). However, biosurfactants have not yet been employed extensively in industries because of their relatively high production cost.

Recently, the production of biosurfactants from microorganisms has been investigated by many researchers (Cirilli and Carman, 1985, Bernheimer and Avigad, 1970, Uchida et al., 1989). However, the purification of the novel biosurfactant from Rhodotorula mucilaginosa KUGPP-1 has not been previously reported. Also, *R. mucilaginosa* could produce some carotenoid pigments in cultured broth without the aggregation of hydrophobic carotenoids. However, the mechanism of the dispersion of carotenoids by *R. mucilaginosa* remains unknown.

In this paper, we found a novel biosurfactant with the astaxanthin-dispersing ability in the cultured broth of *R. mucilaginosa* KUGPP-1. We herein describe the purification of the novel biosurfactant (glycoprotein) from *R. mucilaginosa* KUGPP-1 and characterize it.

**MATERIALS AND METHODS**

**Various yeast strains**


The strain KUGPP-1 used in this study has its origins in the Antarctic origin. For phylogenetic characterization, the 28S rRNA gene was amplified from genomic DNA by PCR using the universal primer pair NL1 (or NL4) and Ready-To-Go PCR Beads. The partial 28S rDNA sequence of strain KUGPP-1 was closest to *R. mucilaginosa* NBRC 0003 (about 100%).

**Media and cultivation**

The cells were maintained as a 20% (v/v) glycerol stock at -80°C after being grown in a YM medium composed of (per liter distilled water, pH 6.5) peptone, 5.0 g; yeast extract, 3.0 g; malt extract, 3.0 g; and glycerol, 5.0 g. Seed cultures were carried out in the YM medium at 18°C or 30°C with reciprocal shaking at 120 rpm for 48 h or 24 h. The pH was adjusted to 6.0 by addition of HCl mol 1⁻ or NaOH mol 1⁻.

**Measurement of the Amounts of Protein and Polysaccharide**

The protein content of the eluted fractions on chromatography was determined according to the BCA method (Hill and Straka, 1988) with a BCA protein Assay kit (PIERCE) using bovine serum albumin as the standard. The total sugar content of the eluted fractions on chromatography was evaluated by the phenol-sulfuric method described by Dubois et al. (1956) using glucose as the standard.

**Purification of the Biosurfactant**

After cultivation on YM medium at 30°C for 48 h, the yeast cells were removed by centrifugation at 10,000 x g for 15 min and aseptic filtration (0.45 μm). The supernatant (10 L) was concentrated by ultrafiltration through a Minitan Ultrafiltration Filter Plate (nominal molecular weight cut off of 100,000, polysulfons, blue, Millipore Co.) to a volume of about 100 ml. The concentrated sample was added to 4 volumes of cold acetone (stored at -80°C), and this mixture was then stirred at 4°C for 30 min. After centrifugation at 10,000 x g for 15 min, the precipitate was suspended in 10 ml of a 20 mM Tris-HCl buffer (pH 8.0) and exhaustively dialyzed against the same buffer to remove the acetone.

The dialysate solution was put onto a DEAE column at a flow rate of 2 ml/min with an AKTA prime system (16 mm × 20 cm, GE Healthcare Co.) previously equilibrated with a 20 mM Tris-HCl buffer (pH 8.0). The adsorbed materials were eluted with a linear 0 to 1 M NaCl gradient in an equilibration buffer. The active fractions that were eluted with approximately 0.3 M NaCl were combined and dialyzed against a 20 mM Tris-HCl buffer (pH 8.0). The dialyzed solution was applied to a Sephacryl S-400 HR column (10 mm × 100 cm, GE Healthcare Co.) equilibrated with a 10 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The active fractions were pooled and dialyzed against a 10 mM potassium phosphate buffer (pH 7.0). The biosurfactant was diluted for use in subsequent studies and stored at 4°C.

**Protein Electrophoresis**

SDS-PAGE of the purified biosurfactant was done using the method of Laemmli (1970) with Ready gel (Bio Rad Co.). Protein was stained using a Silver stain Kit (Wako Co., Japan) and PAS staining (Kapitany and Zebrowski, 1973). For the relative molecular mass measurement, galactosidase (115,660), phosphorylase B (97,316), ovalbumin (53,533), carbonic anhydrase (37,248), soybean trypsin inhibitor (29,385), lysozyme (20,415), and aprotinin (6,919) were used as the standards for PAS staining (BioRad Co.), and myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), bovine serum
albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), lysozyme (14,400), and aprotinin (6,500) were used as the standards for silver staining (BioRad Co.).

**Assay of astaxanthin-dispersing activity**

The sample (0.1 ml) was mixed with 1.8 ml of a 10 mM potassium phosphate buffer (pH 7.0) and 0.1 ml of a 0.02% (v/v) astaxanthin dimethylsulfide (DMSO) solution. This mixture was kept at room temperature for 15 min. Immediately after being mixed, the absorbance at 440 nm of this mixture was measured (0 min absorbance). After being kept for 15 min, the absorbance at 440 nm was measured (15 min absorbance). The astaxanthin-dispersing activity was indicated as the difference (ΔA_{440}) of both absorbance at 0 and 15 min. High dispersal activity is indicated by a small difference value between each absorbance at 0 and 15 min. Enzyme activities are expressed as international units (U), where 1 U corresponds to ΔA_{440} = 0.040.

**RESULTS AND DISCUSSION**

**Astaxanthin-dispersing activity by the extracellular products of various red yeasts**

Astaxanthin-dispersing activity by the extracellular products of various red yeasts was examined. The extracellular products of *R. mucilaginosa* KUGPP-1 that originated in the Antarctic showed dispersal activity (ΔA_{440}<0.041) of astaxanthin. For other strains, their products in the cultured broths of *R. diobovatum* NBR08688, *R. sphaerocarpum* NBR1438 *R. araucariae* NBR10053 and *R. glutinis* var. *glutinis* NBR0395 showed weak dispersal activities (ΔA_{440}>0.041). However, the strains of NBR08871, NBR0388, NBR0559, 0415, NBR0697, NBR0190, and NBR0387 did not show such activity (ΔA_{440}>0.111). As shown in FIG. 1, the cell of strain KUGPP-1, with a diameter of 9 μm, was the budding growth type. The colony was red on YM solid medium. Although this strain was isolated from Vanda Lake of Ross Island in the Antarctic, some red yeasts belonging to the genus *Rhodotorula* were isolated from the Antarctic as psychrophiles or mesophiles (Alchihab et al., 2009). As *R. mucilaginosa* can produce torularhodin with the highest antioxidant potential in the cell membrane, this carotenoid has the ability to scavenge against singlet oxygen and peroxyl radicals in the membrane (Sasaki et al., 2001). However, although *R. glutinis* can produce torularhodin, the astaxanthin-dispersing activity of this yeast was lower than that of strain KUGPP-1. Perhaps the reason for this might be the high UV level in the area of its origin, the Antarctic.

**Growth curve of *R. mucilaginosa* (antarctic origin) KUGPP-1**

The growth curve of *R. mucilaginosa* (antarctic origin) KUGPP-1 was examined. The optimum temperature for growth was 18°C, though this strain could grow at 10 and 30°C. This result suggested that this strain is a psychrotrophic strain isolated in the Antarctic. Experiments were conducted over 64 h with incubation periods at 18°C and 30°C. The astaxanthin-dispersing activity was confirmed in the culture broth after it had been cultured for 48 h in the stationary phase at 18°C. After 48 h of culturing, the dispersing activity in the cultured broth increased in proportion to the amounts of saccharide and protein in the cultured broth.

**Steps in the Purification steps of the biosurfactant from strain KUGPP-1**

The sample dilutions of the cultured broth exhibited the dispersal activity of ΔA_{440} = 0.040 at a concentration of 150 μg/ml (TABLE 1). Also, the total amount of protein and sugar in the cultured broth were 3,149 and 2,075 mg, respectively (TABLE 1). The

**FIG. 1.** Microscope image of the cell and colony photograph of strain KUGPP-1. (a) Microscope (×400) Bar indicates 10 μm. (b) Photograph

**TABLE 1.** Step in the purification step of a novel biosurfactant from strain KUGPP-1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total saccharide (mg)</th>
<th>Total protein (mg)</th>
<th>Dispersing activity ΔA_{440}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>2075.0</td>
<td>3149.0</td>
<td>0.040</td>
</tr>
<tr>
<td>Ultrafiltration (&gt;100,000)</td>
<td>68.0</td>
<td>40.0</td>
<td>0.025</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>39.7</td>
<td>8.9</td>
<td>0.023</td>
</tr>
<tr>
<td>DEAE Toyopearl 650M</td>
<td>3.8</td>
<td>0.9</td>
<td>0.033*</td>
</tr>
<tr>
<td>Sephacryl S400R</td>
<td>2.6</td>
<td>0.3</td>
<td>0.026*</td>
</tr>
</tbody>
</table>

Assay was done at a saccharide concentration of 150 μg/ml.

*At two steps each sample at a saccharide concentration of 10 μg/ml was examined.*
astaxanthin-dispersing activity might be caused by the emulsifying activity like that of biosurfactants. Among some well-known biosurfactants, surfactin produced by \textit{Bacillus subtilis} (Bernheimer and Avigad, 1970), mannosylerythritol lipids produced by \textit{Candida antarctica} (Kitamoto et al., 1993), and succinoyl trehalose lipids produced by \textit{Rodococcus erythropolis} (Uchida et al., 1989), are composed of a protein or peptide and sugar as one portion of a molecule. We assumed that the material having dispersal activity might be peptide, glycoprotein, etc.

One of the biosurfactants, composed of both protein and polysaccharide, was a mannoprotein from \textit{Saccharomyces cerevisiae} (Cameron et al., 1988). Recently, a bioemulsifier from spent yeast obtained from Thai traditional liquor distillation was found to be composed of 96% carbohydrates consisting mainly of glucose with minor quantities of mannose, and 4% of protein built from 17 amino acids (Dikit et al., 2010). As the molecular weights of these glycoproteins as biosurfactants were over 100,000, the effect of ultrafiltration with the condition of the nominal molecular weight of 100,000 as the cut-off on the dispersal activity was examined. Only the ultrafiltrated sample over the molecular weight of 100,000 had dispersing activity. We could predict that this active compound might be a glycoprotein like mannoprotein.

Next the active fraction after treatment with acetone precipitation and dialysis was subjected to DEAE-Toyopearl 650M column chromatography. As shown in Fig. 2, the active compound could adsorb this column and was eluted with a 20 mM Tris-HCl buffer (pH 8.0) containing approximately 0.3 M NaCl. This active fraction was then concentrated with ultrafiltration, and then subjected to gel filtration column chromatography (Fig. 3 (a)). A summary of the steps for the purification of the biosurfactant from strain KUGPP-1 is shown in Table 1.

The purified biosurfactant from 500 ml of the cultured broth was 0.3 mg protein (total amount of saccharide; 2.6 mg). The yields of the biosurfactant judged from total saccharide or protein were 0.12% and 0.009%, respectively. The specific activity (U/mg saccharide) of the purified biosurfactant was

![FIG. 2. Separation of the biosurfactant using DEAE-Toyopearl 650M column chromatography. Closed circles show the absorbance at 490 nm (polysaccharide), open circles the absorbance at 215 nm (protein). The solid line is the concentration gradient of NaCl. The active fraction is the peak with the oblique line.](image)

![FIG. 3. Estimation of the molecular weight using gel filtration chromatography. (a) Gel filtration column chromatograph. Closed circles show the absorbance at 490 nm (polysaccharide), open circles the absorbance at 215 nm (protein). The active fraction is the peak with the oblique line. (b) Calibration curve of the material with a high molecular weight. Open circles indicate the elution volume of the purified biosurfactant. Closed circles indicate the elution volume of standard dextran; Blue dextran; 2,000,000, dextran; 500,000, dextran; 70,000, dextran; 50,000.](image)
200 U/mg. As the specific activity (U/mg) of the cultured broth was 6.67 U/mg, and the total units of the cultured broth and purified biosurfactant were 13340 U and 520 U, and the dispersal activity yield was 3.9%. The purified biosurfactant was diluted and then stored at 4°C for subsequent studies.

**Molecular mass and subunit composition**

The relative molecular mass of the biosurfactant was estimated by gel filtration chromatography (FIG. 3(a)). A relative molecular mass of about 730,000 was calculated from the gel filtration chromatography using some dextran as the standard material (FIG. 3(b)). The average molecular weight of glycoprotein as the bioemulsifier from spent yeast obtained from Thai traditional liquor distillation was 193,000 Da (Dikit et al., 2010). Also, the cell wall mannoproteins that were associated with the expression of foam formation either at the beginning of the fermentation process or at the end in the case of sparkling wines were encoded by the gene involved in foam formation, named FPG1 (Blasco et al., 2011). Although a protein of 770 amino acids with a molecular mass of 72,112 Da was inferred from the DNA sequence, the molecular weight of this mannoprotein was over 100,000. As shown in FIG. 4(b), the purified biosurfactant preparation migrated as a single protein band on SDS-PAGE (13% (w/v) acrylamide) by PAS staining and silver staining. By the application of PAS and silver staining on the sample treated with periodic acid, it was revealed that the carbohydrate components were associated with this protein and the molecular weight of the protein moiety was 29,000.

The amounts of protein and polysaccharide in the purified biosurfactant were examined. This biosurfactant was comprised of polysaccharide and protein, in an approximate molar ratio of 9 : 1. Based on the ratio of protein and sugar and the estimation of molecular weight by gel filtration (FIG. 3), the purified biosurfactant might be a dimer glycoprotein having a molecular weight of approximately 300,000.

The effect of protease treatment on dispersal activity in purified biosurfactant was examined. The treatments by protease from Bacillus subtilis (SIGMA), proteinase K from Trichiria album (SIGMA) and Thermolysin from Bacillus thermoproteolyticus (SIGMA) at 37°C decreased the dispersal activity. This result indicates that the protein moiety in this biosurfactant plays an important role in the expression of the dispersal activity. Candida lipolytica ATCC8662 produced liposan, which was composed of 83% carbohydrate and 17% protein, in the cultured broth of YNB medium supplemented with 1% hexadecane (Cirigliano and Carman, 1985). Although the molar ration of polysaccharide and protein in this biosurfactant was 5 : 1, the purified biosurfactant in this study might be similar to the structure of liposan.

**Sugar composing the novel biosurfactant**

The novel biosurfactant was treated by acid hydrolysis using sulfuric acid for 4 h. The hydrolyzed sample solution was applied to a TSK gel Sugar AXG column (4.6 mm ID x 15 cm) equilibrated with 0.1 M boric acid (pH 8.7). The detection of derivatized sugar was done by examining the fluorescence intensity at an excitation wavelength of 288 nm and an emission wavelength of 470 nm. By HPLC analysis with (Sugar analysis system, Tosoh Co., Japan), the composition of the polysaccharide moiety in the purified biosurfactant was mannose, galactose and glucose. Also, these sugars were found at a ratio of 5

![FIG. 4. SDS-PAGE analysis by PAS and silver staining.](image)

(a) PAS staining (b) Silver staining

lane 1: bovine serum albumin (negative control), lane 2: ovalbumin (positive control), lane 3: purified biosurfactant, lane 4: purified biosurfactant after hydrolyzing with periodic acid.
(mannose) : 4 (glucose) : 3 (galactose). Among the various biosurfactants, liposan was composed of glucose, galactose, galactosamine and galacturonic acid (Cirifiliano and Carman, 1985). The biosurfactant, glycoprotein containing mannose as one of the components was mannanprotein (Cameron et al., 1988). A fraction containing the mannanproteins released during fermentation from the winemaking strain of *S. cerevisiae*, Maurin PDM, was able to reduce the visible protein haze in white wine (Dupin et al., 2000). As *S. cerevisiae* could secrete mannanprotein as the biosurfactant, this strain might secrete glycoproteins with dispersal activity into the culture broth to solubilize and stabilize some carotenoids in the cultured broth.

**Particle size of the micelle of the astaxanthin pigment**

The grain size distribution of the micelles was measured with a dynamic-light-scattering type particle diameter distribution measuring device. The particle size of the micelles was measured 24 hours later after having been mixed with astaxanthin, DMSO, and the biosurfactant (or surfactin Na). The average particle size of the surfactin micelle was about 250 nm. However, the average particle size of the biosurfactant micelle was about 410 nm. This difference in particle size was caused by the different molecular sizes of the biosurfactants.

**Stability of the astaxanthin in the the micelle particles of purified biosurfactant**

The color tone change of the micelle particles of astaxanthin is shown in FIG. 5. As a result, for the astaxanthin in the new micelle particles of the purified biosurfactant, there was almost no change in absorption maximum wavelength and absorbance even after 48 hours. However, as for the astaxanthin in the micelle particles of the surfactin, the absorption maximum wavelength changed remarkably (FIG. 5 (b)). After 16 hr, the absorbance maximum wavelength was exhibited at two peaks, the absorbance at 470 nm, increased by the increase of insolubility in the astaxanthin-dispersing solution with oxidation. As a result, the astaxanthin contained in the micelle particles of the purified biosurfactant was not oxidized, and was stable for 48 h. This result supports that this biosurfactant might be added into culture broth for the protection of carotene in the cultured broth. A high level of dispersing-activity and emulsifying-activity of this glycoprotein against the astaxanthin solution might be associated with the distribution in the cell wall and the interaction with some carotenoids in this yeast. Furthermore, we predict that this dispersal activity is equal to that of mannanprotein having the emulsifying-activity from *S. cerevisiae*.

Biosurfactants have potential applications in agriculture, cosmetics, pharmaceuticals, detergents, personal care products, food processing, textile manufacturing, laundry supplies, metal treatment and processing, pulp and paper processing and paint industries (Banat et al., 2010). Biosurfactants are more effective, selective, environmentally friendly and stable than many synthetic surfactants. The most common biosurfactants are glycolipids in which carbohydrates are attached to a long-chain aliphatic acid. To our knowledge this study represents the first detailed investigation of a polymeric glycoprotein of a biosurfactant to stabilize carotenoid, which has a high level of antioxidation, in solution.

![FIG. 5. Stability of astaxanthin by micelle particles using the biosurfactant. (a) New biosurfactant (b) Surfactin](image-url)
The extracellular products of *R. mucilaginosa* (Antarctic origin) KUGPP-1 and *Phaffia rhodozyma* had a dispersal effect ($\Delta A_{440} < 0.041$) on astaxanthin. However, each strain of NBRC 0871, NBRC0388, NBRC0559, NBRC0415, NBRC0697, NBRC0190, and NBRC0387 did not show such activity ($\Delta A_{440} > 0.111$). The partial 28S rDNA sequence of strain KUGPP-1 showed 100% homology to *R. mucilaginosa* NBRC 0003. The difference in their activities was caused by the difference in the antioxidizing activity of major carotenoids that are distributed in the cell wall. We assumed that the stability of major carotenoids having a high level of antioxidizing activity can be attributed to polysaccharides in the cell wall. The component (49%) in the cell wall of *R. mucilaginosa* was a chitin enriched fraction, which was composed of glucosamine (Bahmed et al., 2003).

The novel biosurfactant was purified from extracts to the ultrafiltration state by acetone precipitation and chromatography on a DEAE-850M and gel filtration on a Sephacryl S-400HR (FIG. 3). The molecular mass of purified biosurfactant was estimated to be about 730,000 by gel filtration chromatography (FIG. 3). The purified biosurfactant was comprised of sugar and protein in an approximate molar ratio of 9 : 1. The sugars were comprised of mannose, glucose and galactose (5 : 4 : 3). The high level of glucose amount might be caused by the chitin as the major component in the cell wall (Bahmed et al., 2003) because the glucose amount in the hydrolyzate of the purified biosurfactant might originate from glucosamine in the chitin.

The shortened locust bean gum (LBG) in the cultured broth after the culture of *Candida utilis* NBRC1086 for 24 h in LBG medium containing 0.5% LBG had emulsifying-activity like some biosurfactants (Kawahara et al., 2008). The mannose/galactose ratio in the acid hydrolysate in shortened LBG was found to be 2.8 : 1. This emulsifying-activity is ascribed to the hydrogen bond between each hydroxyl group of two mannose moieties from different mannan chains. However, the fact that this mannose residue in this biosurfactant is composed of a linear mannan chain without a side chain has been poorly understood. Regardless, this biosurfactant could protect astaxanthin against oxidation during solution stock (FIG. 5). Although the mechanism of stabilization against astaxanthin by the purified biosurfactant remains to be resolved, the purified biosurfactant would be useful as a stabilizing reagent against various antioxidants like the astaxanthin solution with a high level of stability.

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REFERENCES


