Purification and Reconstitution of an Integral Membrane Protein, the Photoreaction Center of *Rhodobacter sphaeroides*, Using Synthetic Sugar Esters

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H. Peters, C. Schmidt-Dannert, L. Cao, U.T. Bornscheuer and R.D. Schmid
University of Stuttgart,
Stuttgart, Germany

ABSTRACT

Detergents are indispensable reagents for the extraction and solubilization of integral membrane proteins, but their removal from a reconstituted phospholipid-protein complex is usually desirable. In this paper, we describe a novel method in which the synthetic sugar esters 6-O-octanoyl- β -D-glucose (OG) or 6-O-octanoyl- β -D-mannose (OM) are used as detergents for both the isolation and the rapid reconstitution of the photosynthetic reaction center protein of *Rhodobacter sphaeroides*. Following solubilization of the reaction center with OG or OM and reconstitution of this protein in liposomes, a convenient removal of these detergents was achieved within less than two hours by hydrolytic cleavage of the sugar esters using immobilized lipases. Best results were achieved with lipase from *Bacillus* sp. immobilized on silica gel.

INTRODUCTION

Before purification, lipid-associated integral membrane proteins must be separated from the lipid membrane components. This step, termed solubilization, is commonly achieved with amphiphilic, anionic or nonionic detergents such as *N,N*-dimethyldodecylamine *N*-oxide (LDAO), cholate, *n*-octyl- β -D-glucopyranoside (OGP), β -dodecyl-maltoside or Triton® X-100 (5,12,14,17). Detergents usually must be present during each purification step to maintain both protein solubility and functional activity but should be removed from the reconstituted proteoliposomes.

Nonionic detergents are often preferred over amphiphilic or anionic surfactants because they are less prone to denature proteins and do not interfere with purification steps involving ion-exchange chromatography. The nonionic surfactant OGP is particularly popular as a detergent for the solubilization of membrane proteins and their reconstitution in liposomes (7,8,10,13,16,20). OGP has a high critical micellar concentration (CMC) (23.3 mM) and thus can be removed by dialysis (18,25). However, a substantial reduction of surfactant may take up to 48 h (5), enough time for a protein to lose activity or undergo denaturation. If nonionic detergents of low CMC such as Triton X-100 (15) are used, the addition of Biobeads-SM2 (11) or Amberlite XAD-2 (19), both of which are commercially available, has proven a useful technique to remove trace levels of surfactants.

In the course of our studies on the synthesis of sugar fatty acid esters by li-

pase catalysis, we have recently synthesized 6-O-octanoyl- β -D-glucose (OG) and 6-O-octanoyl- β -D-mannose (OM) (1,2), which match the surfactant properties of OGP but have advantages for the purification of membrane proteins. Thus, unlike OGP, OG and OM can both be hydrolyzed in a lipase-catalyzed reaction, leaving the phospholipids within the liposomes unaffected. We describe here the application of this technique for the rapid purification and reconstitution of the photosynthetic reaction center protein from *Rhodobacter sphaeroides*.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and LDAO were purchased from Fluka Chemie (Buchs, Switzerland). OGP was purchased from Sigma (St. Louis, MO, USA), and silica gel was purchased from Merck (Darmstadt, Germany). OG and OM were synthesized as previously described (4). Lipase from *Bacillus* sp. was obtained from Asahi Chemicals (Tokyo, Japan), and lipase from *Candida rugosa* was purchased from Biocatalyst (Pontybridd, UK). Immobilized lipases from *C. antarctica* (CAL-B = SP435, CAL-A/B = SP382) and from *Rhizomucor miehei* (Lipozyme) were gifts from Novo Nordisk (Bagsvaerd, Denmark) and Roche Diagnostics (Penzberg, Germany). All other chemicals were from common suppliers and obtained at the highest purity available.

Cultivation of *R. sphaeroides*

R. sphaeroides DSM 158 was cultivated in 1-L shaking flasks in a medium described by Sistrom (21). To obtain anaerobic conditions, cultures were aerated with nitrogen and cultivated in darkness for 16 h before illumination with 1300 lux for three days. Cells were harvested in the late log phase by centrifugation (11 000× *g* at 4°C) and frozen at -20°C for storage or directly used for isolation and purification of the photoreaction center protein.

Extraction and Purification of the Reaction Center Protein

R. sphaeroides cells (100 g) were suspended in 200 mL 20 mM Tris-HCl (pH 8.0) containing 10 mg lysozyme and DNase. The suspension was stirred for 45 min in darkness at room temperature. Following sonification of the suspension for 60 min on ice, the debris was removed from the preparation by low-spin centrifugation at 20 000× *g* for 30 min at 4°C. Chromatophores in the supernatant were collected by ultracentrifugation at 200 000× *g* for 2 h at 4°C. Extraction of the reaction center protein from the chromatophores was performed by diluting the chromatophores to $A_{850} = 1$ [850 nm = Qy-band of special pair, bacteriochlorophyll dimer (23) with 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM PMSF containing either 8 mg/mL sugar esters (OG or OM) or 0.25% (v/v) LDAO, followed by agitation for 1 h at room temperature]. The solubilized reaction center protein was separated from chromatophores by ultracentrifugation at 200 000× *g* for 2 h at 4°C. The chromatophores were then extracted a second time.

Purification of the solubilized reaction center protein was carried out as previously described (16) by ion exchange chromatography using S- and Q-Sepharose® columns sequentially. The columns (30 × 2 cm) were equilibrated with 20 mM Tris-HCl (pH 8.0) containing 8 mg/mL sugar ester or 0.25% LDAO (v/v), respectively. The extracted reaction center protein was loaded on the S-Sepharose column with 250 mL equilibration buffer (flow rate of 4 mL/min) and was retained on the

Q-Sepharose column. After disconnection of the S-Sepharose column, the reaction center protein was eluted from the Q-Sepharose column with a gradient of 0.125–1 M NaCl in equilibration buffer. The reaction center protein containing fractions were pooled, concentrated by ultrafiltration and finally dialyzed against equilibration buffer.

The purity of the reaction center protein was estimated by SDS-PAGE, followed by staining with Coomassie Blue®. The reaction center protein concentration was determined spectrophotometrically at 802 nm ($\epsilon = 288 \text{ mM/cm}$) (22). The absorbance ratio A_{280}/A_{802} was routinely used for quantification of purity. Protein concentrations were determined with bicinchoninic acid, using the BCA Protein Detection Kit from Pierce Chemical (Rockford, IL, USA).

Immobilization of Lipases

Immobilization of lipases on activated silica gel was performed according to a modified method from Weetal (24) or on anion exchange resins Q-Sepharose or *n*-octyl-Sepharose as described elsewhere (3).

Hydrolysis of Sugar Fatty Acid Esters

Immobilized lipases (100 mg) (*Bacillus* sp. or *C. rugosa*) or 20 mg commercially available immobilized lipases (CAL-B, CAL-A/B or RML) were added to 1 mL 50 mM Tris-HCl buffer (pH 8.0) containing 9.2 mg OG. The reaction mixture was incubated at 40°C, and 100-μL aliquots were withdrawn at intervals of 1 h and analyzed by TLC and HPLC (data not shown).

Lipase Method for Reconstitution

DMPC (1 mg/mL) was solubilized with 8 mg/mL OGP in 100 mM Tris-HCl (pH 8.0). Then, the purified reaction center protein solubilized in OG (final molar ratio of protein:lipid of 1:1000) and 20 mg/mL immobilized lipase were added, and the mixed micellar solution was incubated at 40°C for 0.5–1 h. The immobilized matrix was separated from the liposome solution by short centrifugation or filtration.

Analytical Methods

TLC analysis was performed on nonactivated silica gel plates using ethylacetate:methanol:water (80:20:5) as the developing system and OG as a standard. The plates were sprayed with 50% (v/v) sulfuric acid and heated at 110°C for 5 min to develop the sugar and sugar ester spots.

Quantitative analysis of OG conversion was carried out on an HPLC system (Sykam, Gilching, Germany) equipped with a Nucleosil 120-5C₁₈ column (Crom, Herrenberg, Germany) and a light-scattering detector. Chromatography was performed with methanol:acetonitrile:water (50:30:20) as the mobile phase and a flow rate of 1 mL/min at 40°C.

The integrity of liposomal phospholipids after lipase treatment was checked by HPLC by extracting the lipid components from the reaction mixture using chloroform:methanol (2:1) followed by washing steps with sodium bicarbonate and water. Analysis was carried out on a 250 × 4-mm I.D. column (Macherey-Nagel GmbH, Düren, Germany) packed with 5 μm unmodified Nucleosil. Elution (flow rate of 1 mL/min) was performed in gradient mode (starting with 100% A and decreasing over 20 min to 100% B) with solvent A = isopropanol:isohexane (4:3) and solvent B = isopropanol:isohexane:water (8:6:1.5).

Characterization of Liposomes

Light-scattering measurements of liposomes, diluted with the corresponding buffer to a final phospholipid concentration of 1 mg/mL, were carried out with a Zetasizer 3 particle analyzer (Malvern Instruments, Malvern, UK). The distribution of particle sizes was measured in the range of 3 nm–6.54 μm using dynamic light scattering.

RESULTS

Solubilization and Purification of Reaction Center Protein

The reaction center protein was efficiently extracted from the chromatophores with both OG or OM (Table 1).

Research Report

Yields of the protein were similar if either LDAO or OG was used, but dropped to half if extractions were based on OM. However, the purification factor using OM was about 5.5 times higher as compared to the LDAO extractions.

Extracts containing the protein, solubilized with either one of the three detergents, were further purified by ion exchange chromatography, maintaining the same detergent concentrations used for extraction throughout the entire chromatographic procedure. Instead of the three chromatographic steps used in the protocol of Gray et al. (9), we developed a single-step purification using S- and Q-Sepharose columns in sequence. While the reaction center protein was retained on the Q-Sepharose column at pH 8.0, most other proteins either passed through both columns unbound or bound firmly to the S-Sepharose column. By detaching the S-Sepharose column, the protein could be eluted from the Q-Sepharose column using a NaCl gradient from 0.125–1 M. Elution of the protein started at 0.4 M NaCl, whether or not LDAO or sugar esters were used during the chromatography. The protein-containing fractions were pooled and analyzed (Table 1). In the case of LDAO and OG, about 80% of the protein applied to the ion exchange columns was finally eluted from the Q-Sepharose column, while in the case of OM, a somewhat lower yield of about 70% was obtained.

The purity of the reaction center protein solubilized and purified by means of the sugar esters was higher (1.7 or 1.9) (Figure 1) compared to the values obtained using LDAO (1.2) as the detergent (Table 1).

Hydrolysis of Sugar Fatty Acid Esters with Immobilized Lipases

To achieve reconstitution of pure reaction center protein, the detergent must be removed during proteoliposome formation. Because both OG and OM were synthesized by lipase-catalyzed esterification, it was likely that lipase-catalyzed hydrolysis of these esters should allow for the complete hydrolysis of these surfactants. The use of lipases in an immobilized form should further ensure a simple removal of the

Table 1. Isolation and Purification of the Photosynthetic Reaction Center Protein from *R. sphaeroides* with 8 mg/mL Synthetic Sugar Esters OG or OM or 0.25% (v/v) LDAO

Purification step	Yield (%)	Purification ratio (A_{280}/A_{802})
Cell disruption	100	20
Chromatophores	47	3.1
Extractions		
LDAO	14.8	1.7
OG	12.9	2.4
OM	7.6	7.6
Ion exchange chromatography		
LDAO	11.8	1.2
OG	10.2	1.7
OM	5.2	1.9

biocatalyst after completion of the reaction. Hence, commercial lipases from either *C. rugosa* or *Bacillus* sp. were covalently immobilized on activated silica gel, and *C. rugosa* was also immobilized on *n*-octyl- and Q-Sepharose. In addition, commercial immobilized lipases from *R. miehei* (Lipozyme) and *C. antarctica* (CAL-B, CAL-A/B) were investigated. Since OG, when compared to OM, led to somewhat higher yields of reaction center protein after extraction and purification, hydrolysis of OG was chosen as the model reaction for the selection of an appropriate immobilized biocatalyst. After 2 h of incubation, greater than 60% of the sugar ester was hydrolyzed with all immobilized lipase preparations, except for *C. rugosa* immobilized on Q-Sepharose. Complete hydrolysis after 2 h of incubation was obtained only with *C. rugosa* immobilized on *n*-octyl-Sepharose and with *Bacillus* sp. immobilized on silica gel (Table 2).

Liposome Preparation by Hydrolysis of Sugar Fatty Esters

For liposome preparation, a mixture consisting of the commercially available DMPC (1 mg/mL) solubilized with either OG or OM (8 mg/mL) and immobilized lipase (100 mg/mL) in 100 mM Tris-HCl (pH 8.0) was incubated for 2 h at 40°C. Liposomes were then separated by a brief centrifugation or filtration step, which worked well if silica was used as an immobilization

matrix, but failed with lipases immobilized on other matrices, possibly because of liposome adsorption. *Bacillus* sp. immobilized on activated silica gel (100 mg/mL) allowed for the most rapid hydrolysis of sugar esters without interference with liposome formation and thus was chosen for all subsequent experiments.

After 2 h of incubation, the sugar esters used for the solubilization of DMPC were completely hydrolyzed by immobilized *Bacillus* sp., as verified by TLC. Octanoic acid has a solubility of about 7 g/L, and glucose is infinitely soluble in water. As the initial concentration of OG or OM is 8 g/L, we

Figure 1. SDS-PAGE of solubilized and purified reaction center protein after ion-exchange chromatography. Lane 1: low molecular weight marker, lane 2: solubilized protein with OG, lane 3: purified protein in the presence of OM, lane 4: purified protein in the presence of OG. The protein is composed of three subunits M (30.7 kDa), H (28.1 kDa) and L (26.0 kDa).

Table 2. Hydrolysis of OG with Immobilized Lipases

Lipase	Carrier for Immobilization	Conversion (%)		
		1 h	2 h	3 h
CRL ^a	<i>n</i> -octyl-Sepharose	85	100	-
CRL ^a	Q-Sepharose	3	7	10
CRL ^a	Silica	40	75	90
BSL ^a	Silica	80	100	-
CAL-B ^b	Polyacrylic resin	38	77	80
CAL-A/B ^b	Polyacrylic resin	30	62	76
RMLb	Duolite 568	47	60	74

Addition of ^a100 mg/mL wet immobilized or ^b20 mg/mL dry immobilized lipase.
 Reaction conditions: 9.2 mg/mL OG in 100 mM Tris-HCl (pH 8.0) at 40°C.
 CRL, *C. rugosa*; BSL, *Bacillus* sp.; RML, *R. miehei*.

presume in the absence of experimental evidence that glucose has dissolved completely in the aqueous phase and trace octanoic acid may have remained

in the liposomes. DMPC, the phospholipid used for liposome formation, did not react with BSL, as confirmed by HPLC analysis.

The liposomes, spontaneously formed after hydrolysis of the sugar esters, were easily separated from the silica gel matrix by filtration or a short centrifugation step. Light-scattering measurements revealed the presence of rather large vesicles of 170–610 nm (OG) and 145–710 nm (OM) (Table 3). To obtain a narrower particle-size distribution, the suspension was extruded 10 times through a polycarbonate membrane with a pore diameter of 200 nm. After this step, DMPC liposomes prepared by hydrolysis of either sugar ester became more uniform with a mean diameter of about 150 nm, as confirmed by light-scattering measurements (Figure 2) and were similar to DMPC liposomes prepared by the conventional detergent dialysis method using OGP as a detergent (mean diameter of about 203 nm) (data not shown).

Similar results were achieved when DMPC was replaced by DPPC. Again,

Research Report

Table 3. Liposome Size as Determined by Light Scattering

Sugar fatty ester	Phospholipid	Extrusion (200-nm pore diameter)	Light scattering
OG	DMPC	-	110–850
	DMPC	10 ×	110–280
	DMPC + RC	10 ×	130–225
	DPPC	10 ×	145–210
	DMPC	-	120–810
OM	DMPC	10 ×	118–221
	DMPC + RC	10 ×	165–197
	DPPC	10 ×	135–168

RC, reaction center protein.

spontaneous formation of DPPC liposomes occurred after 2 h of incubation, and a homogenous liposome population with a mean diameter of 150 nm formed after extrusion (Table 3). In addition, we found that *Bacillus* sp. could be reused 10 times without activity loss (data not shown).

Reconstitution of Reaction Center Protein into Liposomes

For our reconstitution experiments, reaction center protein, solubilized by

OG, was added to a mixture of DMPG/OG (1:8, w/w) to a final concentration of protein:DMPC of 1:1000, and immobilized lipase was added. After 2 h, formation of large proteoliposomes with diameters of 700 nm was observed by light-scattering measurements. Again, extrusion led to a rather homogenous population of proteoliposomes with a mean diameter of 150 nm (Table 3). A continuous absorption spectrum in the range of 200–900 nm for these proteoliposomes showed a typical increase of absorption at lower

Figure 2. Light-scattering measurements of DMPC-reaction center protein liposomes formed after *Bacillus* sp.-catalyzed hydrolytic removal of OG. Particle size distribution of liposomes was determined directly after hydrolysis (solid line) and after extrusion ten times through a 200-nm polycarbonate membrane (dotted line).

wavelengths because of light scattering and the adsorption of incorporated protein (data not shown). With both sugar esters, 34%–39% of the protein initially added to the reaction mixture was incorporated into DMPC liposomes formed during lipase hydrolysis of OG, as calculated by measuring the absorbance of the proteoliposomes at 802 nm.

DISCUSSION

We report here that synthetic sugar fatty esters such as OG and OM allow for the solubilization of the reaction center protein of *R. sphaeroides* in yields and purities comparable to LDAO (6). The advantage of using these compounds is that they can be removed by lipase-catalyzed hydrolysis and thus allow for a simplified purification scheme. Using this method, the reconstitution of the reaction center protein of *R. sphaeroides* into DMPC phospholipid vesicles could be achieved in yields greater than 30%. Investigations are now underway to investigate the use of this method for the purification and reconstitution of other membrane proteins.

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Address correspondence to:

Prof. Rolf D. Schmid
Institut für Technische Biochemie
Universität Stuttgart
Allmandring 31
D-70569 Stuttgart
Germany
Internet: rolf.d.schmid@rus.uni-stuttgart.de