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THE USE OF OCTYL β-D-GLUCOSIDE AS DETERGENT FOR HOG KIDNEY BRUSH BORDER MEMBRANE

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Summary

Octyl β-D-glucoside was synthetized from α-acetobromoglucose with an improved method yielding a very pure product with a sharp melting point (108—109°C) and free of intermediate products as judged by IR and NMR spectra. The yield of the synthesis is 66% when referred to α-acetobromoglucose. The potency of this compound as a detergent on hog kidney brush border membranes was compared to the action of Triton X-100. Octyl glucoside preferentially extracts aminopeptidase M and γ-glutamyltranspeptidase in a concentration-dependent manner. The more deeply imbedded membrane enzyme, alkaline phosphatase, was relatively resistent to the action of octyl glucoside. In contrast, Triton X-100 extracted all membrane proteins to about the same extent. Additionally it was found that octyl glucoside can be removed from membrane extracts by Biobead SM 2. The capacity of the beads is about 170 mg detergent/g of dry Biobead SM 2. Thus octyl glucoside seems to be a useful tool for solubilization and purification of brush border membranes proteins.

Introduction

In 1975 Baron and Thompson [1] introduced octyl β-D-glucoside as a new non-ionic detergent for membrane research. The great advantage of octyl β-D-glucoside over the commonly used Triton X-100 is its high critical micelle concentration which allows a more rapid removal of the detergent from membrane extracts by dialysis. In addition, the compound proved to be superior to Triton X-100 in several instances by allowing a better preservation of the biological activity of extracted membrane proteins [1,2]. These reasons led us to the use of octyl β-D-glucoside as a solubilizing agent for the isolation of components

from renal brush border membranes. Our studies show that octyl β-D-glucoside is a potent detergent for brush border membranes with selectivity different from that of Triton X-100.

Materials and Methods

Materials. α-Acetobromoglucose (stabilized with 2% $CaCO_3$), *n*-octanol, organic solvents, sodium methylate, $LiAlH_4$ and silver nitrate were purchased from Merck (Darmstadt, F.R.G.) or Merck-Schuchardt (München, F.R.G.), Triton X-100, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), glycylglycine, L-glutamyl-4-nitroanilide and tris(hydroxymethyl)aminomethane (Tris), were obtained from Serva (Heidelberg, F.R.G.) and Biobead SM 2 from Bio. Rad. (München, F.R.G.).

Instruments. The melting points (uncorrected) were determined with SMP-20 from Büchi Laboratoriums-Technik AG (Switzerland). The nuclear magnetic resonance spectra (NMR) were recorded with the NMR-spectrometer, WH 270 (270 MHz) Bruker Physik AG (F.R.G.) or with T 60 Varian (U.S.A.). The substances for measurement were usually dissolved in deuterated chloroform and tetramethylenesilan was used as internal standard.

Infrared spectra of compounds were recorded with type 457, Perkin-Elmer (U.S.A.). The substance was dissolved in a drop of chloroform and squeezed between flat plates of sodium chloride (transparent throughout the 4000—667 cm^{-1} region). The optical rotation was measured in chloroform at 22°C with No. 141 polarimeter from Perkin-Elmer (U.S.A.).

Ultracentrifugation was carried out with L3-50 ultracentrifuge and swinging bucket rotor SW 50.1 or SW 27 from Beckman Instruments GmbH (München, F.R.G.).

Synthesis of n-octyl β-D-glucopyranoside. *n*-Octyl β-D-glucopyranoside was synthetized from α-acetobromoglucose with octyl tetra-*O*-acetyl-β-D-glucoside as intermediate product. The original synthesis procedures from Noller and Rockwell [3] were modified in the following ways:

(a) For the preparation of octyl tetra-*O*-acetyl-β-D-glucoside the following reaction mixture was used: α-acetobromoglucose 8 g, *n*-octanol 25 ml, ether 150 ml, silver oxide 5 g; α-acetobromoglucose was recrystallized from ether/petrolether 40 in order to remove calcium carbonate which is used as stabilizer in the commercially available α-acetobromoglucose. A white substance with a sharp melting point of 89°C was isolated. *n*-Octanol was always kept on molecular sieve before use. The last trace of water was removed from the ether by adding $LiAlH_4$ (500 mg/200 ml ether) followed by filtration through an aluminium oxide column (50 g, neutral, activated grade I). Silver oxide was freshly prepared from silver nitrate solution in deionized water (5 g/20 ml H_2O) by adding 2 mol/l sodium hydroxide. The precipitate (Ag_2O) was washed with deionized water until neutral and activated at 150°C for 4 h. The reaction of α-acetobromoglucose with *n*-octanol was complete after approximately 5 h at room temperature. The $AgNO_3$ test [3] was already negative after 3 h.

After the reaction was completed, the ether was evaporated. Most of the octanol was removed at 100—110°C under vacuum (0.1—0.2 mmHg). The resin-like substance was exposed to petrolether 40 (200 ml) with vigorous

stirring for 30 min. After incubation at −20°C overnight the petrolether was decanted. The residue was washed twice again with petrolether and finally dissolved in methanol. Then water was added to the methanol solution until it became cloudy; the cloudy solution was cooled down gradually from 0°C to −20°C and kept at −20°C for 2 days. A white solid material could be isolated with a melting point of 59.5—60°C. 98% of the α-acetobromoglucose could be recovered as octyl tetra-*O*-acetyl-β-glucoside.

(b) The hydrolysis of octyl tetra-*O*-acetylglucoside to octyl β-D-glucoside was carried out in absolute methanol and $2 \cdot 10^{-3}$ M sodium methylate for 2 h under reflux. Methanol was evaporated and the product was crystallized from acetone/petrolether. Melting point of octyl β-D-glucoside: 108—109°C (yield: 66% of the theory, based on α-acetobromoglucose). The sodium methylate solution ($2 \cdot 10^{-3}$ M) was kept in a three-necked flask under nitrogen, in order to exclude moisture and air from the solution.

Preparation of brush border membranes from hog kidney cortex. Fresh pig kidneys were obtained from the local slaughterhouse. Brush border membranes were prepared according to the method described by Vannier et al. [4]. After the 'alkaline treatment', the resulting pellet was resuspended in the solubilization buffer (150 mM KCl, 10 mM $MgSO_4$, 5 mM Hepes, 0.2 mM dithiothreitol, pH adjusted to 7.4 with Tris). In the brush border preparation alkaline phosphatase was enriched 9.8 fold (±0.7; $n = 3$) compared to the homogenate. Contamination with basal-lateral membranes, mitochondria and endoplasmic reticulum was insignificant.

Solubilization of brush border membrane proteins. Membranes were mixed with an equal volume of the detergent solution, e.g. 2% octyl glucoside in the solubilization buffer or 0.2% Triton X-100 in the same buffer, and incubated with stirring at 4°C for 30 min. The membrane suspension was then centrifuged for 15 min at 30 000 × *g* and 4°C leading to a pellet P_1 and a supernatant S_1. The low speed supernatant S_1 was centrifuged for 1 h at 100 000 × *g* and 4°C. The high speed supernatant S_2 is referred to as solubilized membrane protein fraction.

Determination of protein and enzyme activities. Protein. Protein measurements were carried out following the method of Lowry et al. [5] after precipitation of the membrane protein by 10% (w/v) ice-cold trichloroacetic acid.

Alkaline phosphatase (EC 3.1.3.1). Alkaline phosphatase activities were determined using an optimized kinetic test (Merckotest 3344, Merck AG, Darmstadt, F.R.G.).

Aminopeptidase M (EC 3.4.11.2). The activity of the enzyme was measured in the same way as previously reported [6].

γ-Glutamyltranspeptidase (EC 2.3.2.2). γ-Glutamyltranspeptidase activity was measured at 25°C and 405 nm using a Beckman 25 photometer equipped with a recorder. The reaction mixture consisted of 750 μl Tris buffer (175 mM, pH 8.25), in which the substrates (3.9 mM L-γ-glutamyl-*p*-nitroanilide and 39 mM glycylglycine, final concentrations) were dissolved and 50 μl of the sample. The appearance of *p*-nitroaniline was monitored.

Protein and enzyme activity measurements were carried out in duplicate.

Determination of octyl glucoside. Octyl glucoside was determined as glucose with the anthrone method [7]. For the calculation it was assumed that the

sugar component represents 58% of the weight of octyl glucoside. Measurements were carried out in duplicate.

Determination of the binding capacity of Biobead SM 2 for octyl glucoside. In order to estimate the capacity of the Biobeads a batch method was used: 100-mg and 200-mg portions of wet Biobead, with a water content of 61.6%, were stirred in solubilization buffer with varying amounts of octyl glucoside at room temperature until equilibrium was reached, (usually for 1 h); the Biobeads were allowed to settle down and the free detergent in the supernatant was determined by the anthrone method [7].

Results and Discussion

Synthesis of octyl β-D-glucoside

In Table I the physical properties of octyl β-D-glucoside (III), synthetized according to the modified procedures (see Materials and Methods), of α-acetobromoglucose (I), and of the intermediate product octyl tetraacetyl-β-D-glucoside are given. Infrared spectroscopy and NMR data (see Table I) confirm the theoretically expected course of reaction with respect to the presence of C=O band and acyl protons of the acetyl residues in I and II, but not in III, the appearance of octyl protons in II and III, and the presence of free OH groups in III after hydrolysis of octyl tetraacetyl-β-D-glucoside to octyl glucoside. In the NMR spectra a ratio of acyl protons (2.0—2.1 ppm) to octyl protons (0.8—1.7 ppm) of 1.4 was found for octyl tetraacetyl-β-D-glucoside, this value agrees very well with the theoretical value of 1.41 and thus indicates a high purity of the intermediate product. The sharp melting point, the optical rotation and the absence of impurities in the infrared and NMR spectra are the essential criteria for purity of the final product. Concerning the configuration of the glucoside (β or α) the NMR coupling constant at 4.3 ppm (J = 8.0 Hz) corresponds to the theoretical value for β-configurated glucose derivatives [8] and is similar to the one reported by Keana [9].

With the synthesis described above a reproducible yield of 66% based on α-acetobromoglucose for the final product, octyl β-D-glucoside, was obtained. A yield of 60% for the same product based on n-octanol (35% based on α-acetobromoglucose) was reported by Keana [9] and a yield of 40—60% for

TABLE I

SOME PHYSICAL DATA OF GLUCOSE DERIVATIVES

I, α-acetobromoglucose, II, octyl tetraacetyl β-D-glucoside and III, n-octyl β-D-glucoside.

NMR (α ppm)		I	II	III
Octyl protons		absent	0.8—1.7	0.8—1.7
Acyl protons		2.0—2.1	2.0—2.1	absent
Infrared spectroscopy (cm^{-1})	C=O band	1750	1750	absent
	OH band	absent	absent	3400—3500
Melting point (°C)	89		59.5	108—109
Specific rotation $[\alpha]_D^{22}$		+ 195.2	—20.9	—37.1

octyl tetra-*O*-acetyl-β-D-glucoside based on α-acetobromoglucose was given by Noller and Rockwell [3]. Our modified procedure yields 98% of octyl tetra-*O*-acetyl-β-D-glucoside. Thus the yield of both the intermediate product and the final product are considerably higher than those described hitherto in the literature. The main reason for this improvement is the removal of the stabilizer $CaCO_3$ from acetobromoglucose and the exclusion of water moisture from the reaction mixture. In the presence of H_2O and the stabilizer no octyl glucoside was obtained after hydrolysis with sodium methylate although the $AgNO_3$ test [3] indicated a replacement of the bromine atom from the acetobromoglucose molecule in the first reaction step. It was also found that exchange of chlorine against bromine in the α-position of the glucose molecule reduced the reactivity. No reaction product was obtained under identical conditions, when α-acetobromoglucose was replaced by α-acetochloroglucose.

Interaction of octyl glucoside with Biobeads SM 2

1. Binding of octyl glucoside to Biobead SM 2. The binding capacity of Biobead SM 2 for octyl glucoside is presented in Fig. 1. From the linear part of the Eadie-Hofstee plot [10] a capacity of 6.5 mg/100 mg of wet Biobead and 12 mg/200 mg can be evaluated, which corresponds to a capacity of about 170 mg detergent/g of dry Biobead SM 2. The binding is essentially complete after 1 h.

According to the studies of Holloway [11], the capacity of Biobead SM 2 for Triton X-100 is approximately 70 mg/g wet beads, a value which corresponds closely to the value found for octyl glucoside. If, however, the different molecular weights of the two detergents are considered (2800 for Triton X-100 and 290 for octyl glucoside) ten times more molecules of octyl glucoside are bound than of Triton X-100.

2. Removal of octyl glucoside by Biobead SM 2 columns. Commercially

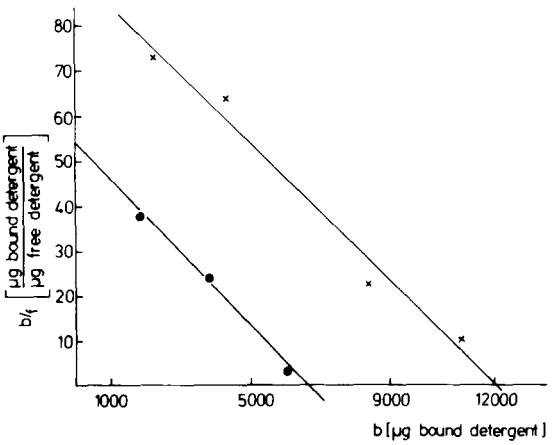

Fig. 1. Eadie-Hofstee plot for determination of binding capacity of Biobead SM 2 for octyl glucoside. 100 mg wet Biobeads (●———●) and 200 mg wet Biobeads (X———X) were equilibrated with various amounts of octyl glucoside in solubilization buffer. The Biobeads were then separated from the supernate by sedimentation and the octyl glucoside content in the supernate was determined as described in Materials and Methods.

available Biobead SM 2 columns (content 1 g Biobead) were used as a routine method for removal of the detergent out of a protein-containing solution. The detergent concentrations are reduced in the following manner and the removal is accomplished without significant loss of enzyme activities. After passage of a 1% octyl glucoside solution (1 ml solution containing 10 mg detergent) through one column 16.3% ± 7.6 of the initial detergent is left. Passages through two more columns reduce the detergent to 5.37% ± 1.4 and 1.8% ± 0.61 of the initial amount (mean values of three experiments ± S.D.) the final concentration is 0.0018% (4 ml containing 0.72 mg octyl glucoside).

In similar studies performed by Holloway [11] with Triton X-100 and deoxycholate the final concentration of the detergent was 0.01% and 0.17%, respectively, i.e. very similar to the critical micelle concentration for these substances. This finding led Holloway [11] to the suggestion that the polymer beads were absorbing only the micelle species of the detergent and not the monomer. For octyl glucoside this seems not to be the case, the final concentration of 0.018% is much lower than the critical micelle concentration of 0.7%. Therefore one can assume that the micellar species as well as the monomers of octyl glucoside interact with the hydrophobic gel matrix; this assumption can also explain the higher capacity of Biobeads SM 2 for octyl glucoside. For the latter fact probably also factors such as size of micelles and of the detergent molecule have to be considered.

Solubilization of porcine kidney brush border by octyl glucoside

In Table IIa, the solubilization of the brush border membrane by increasing concentrations of octyl β-D-glucoside is shown. Solubilization was determined by measuring the appearance of protein and enzymes in the 100 000 × *g* per h supernate, the 'soluble' fraction of the membrane. Below the critical micelle concentration of 0.7% octyl glucoside γ-glutamyltranspeptidase and aminopeptidase M are extracted to the same extent as the membrane protein, above the critical micelle concentration almost 90% of the activity of the two enzymes initially present in the membranes is found in the 100 000 × *g* per h supernate, but only 60% of the membrane protein. Alkaline phosphatase is

TABLE IIa

SOLUBILIZATION OF HOG KIDNEY BRUSH BORDER MEMBRANE PROTEINS WITH DIFFERENT CONCENTRATIONS OF OCTYL GLUCOSIDE

The ratio of detergent to protein was kept constant at 4:1. The results are given as percent of the amount of protein or enzymes initially present in the brush border membranes. The overall recovery for enzymes and protein was 90—100%.

Detergent concentration (%)	% solubilized protein and enzyme			
	Protein	Alkaline phosphatase	Amino peptidase M	γ-Glutamyl-transpeptidase
0.3	14	3	6	5
0.75	56	20	45	45
1.0	63	33	85	82
1.2	70	34	80	87
1.8	80	58	88	97

TABLE IIb

EFFECT OF DETERGENT/PROTEIN RATIO FOR SOLUBILIZATION OF HOG KIDNEY BRUSH BORDER MEMBRANE PROTEINS WITH OCTYL GLYCOSIDE

The final concentration of octyl glucoside was kept constant at 1%, the ratio of octyl glucoside to membrane protein was varied from sample to sample (2:1, 4:1, 6:1, 10:1). For further details see Table IIa.

mg detergent/mg protein	% solubilized protein and enzymes			
	Protein	Alkaline phosphatase	Amino-peptidase M	γ-Glutamyl-transpeptidase
2	47	10	45	40
4	63	33	85	82
6	60	40	93	95
10	63	35	95	95

quite resistant to the action of octyl β-D-glucoside. Only at a very high concentration (1.8%) more than 50% of this enzyme appear in the soluble fraction. The same pattern is observed when the detergent/protein ratio is increased keeping the concentration of octyl β-D-glucoside constant (Table IIb). Again γ-glutamyltranspeptidase and aminopeptidase are extracted to a higher extent (almost 100%) than the protein and the alkaline phosphatase. This results, as

TABLE III

SPECIFIC ACTIVITIES AND ENRICHMENT FACTORS OF ALKALINE PHOSPHATASE, AMINOPEPTIDASE M AND γ-GLUTAMYLTRANSPEPTIDASE AFTER EXTRACTION OF HOG KIDNEY BRUSH BORDER MEMBRANES WITH OCTYL GLUCOSIDE OR TRITON X-100

The specific activity (s.a.) is given in mU/mg protein, the enrichment factor (e.f.) is defined as the ratio between the specific activity found in the fraction under consideration and the specific activity found in the membrane suspension (after addition of the detergent). P_1 is pellet centrifuged at 30 000 × *g* for 15 min; P_2 is pellet centrifuged at 100 000 × *g* for 1 h; S is supernate obtained after centrifugation at 100 000 × *g* for 1 h.

Sample	1% octyl glucoside (detergent/protein 4:1)					
	Alkaline phosphatase		γ-Glutamyltranspeptidase		Aminopeptidase M	
	s.a.	e.f.	s.a.	e.f.	s.a.	e.f.
Membrane suspension	2125	—	1512	—	981	—
P_1	3902	1.84	329	0.22	275	0.28
P_2	6129	2.88	479	0.32	617	0.63
S	935	0.44	2230	1.47	1383	1.41
	0.1% Triton X-100 (detergent/protein 4:1)					
	Alkaline phosphatase		γ-Glutamyltranspeptidase		Aminopeptidase M	
	s.a.	e.f.	s.a.	e.f.	s.a.	e.f.
Membrane suspension	3524	—	1770	—	1015	—
P_1	2270	0.64	140	0.08	98	0.10
P_2	6453	1.83	234	0.13	245	0.24
S	3534	1.00	2030	1.15	1117	1.10

TABLE IV

SOLUBILIZATION OF HOG KIDNEY BRUSH BORDER MEMBRANE PROTEINS BY TRITON X-100

Approximately 15 mg brush border membrane protein (0.25 mg/ml at 0.1% Triton X-100) were solubilized in solubilizing buffer at different detergent concentrations, while the ratio of detergent to protein was kept constant at 4:1. The results are given as percent of the amount of protein and enzymes initially present in the brush border membranes. The overall recovery for enzymes and protein was 85—105%.

Detergent concentration (%)	% solubilized protein and enzymes			
	Protein	Alkaline phosphatase	Amino-peptidase M	γ-Glutamyl-transpeptidase
0.02	14	10	20	10
0.05	80	59	69	94
0.1	87	63	80	91

shown in Table III, in an enrichment of aminopeptidase M and γ-glutamyltranspeptidase in the supernate and of alkaline phosphatase in the remaining membrane fragments. As shown in Table IV, this behaviour is in contrast to the action of Triton X-100 on the brush border membrane. At all Triton X-100 concentrations investigated, there is an almost uniform extraction of protein, alkaline phosphatase, aminopeptidase M and γ-glutamyltranspeptidase. Accordingly no appreciable increase in the specific activity of any enzyme is observed in the solubilized fraction (Table III).

The different action of octyl glucoside and Triton X-100 on the brush border enzymes is difficult to explain, since solubilization of the membranes involves a variety of intermediate reactions such as binding of the detergent to the membranes, formation of lipid-detergent and lipid-protein-detergent micelles and finally delipidation of the proteins [12,13]. At all stages, differences between the two detergents can be expected as already evident from the different interaction of the detergents with the hydrophobic Biobead matrix. It is remarkable, that all the enzymes extracted preferentially by octyl glucoside, belong to the class of brush border proteins, in which the majority of the polypeptide chain is located on the outer surface of the membrane and can also be removed in an enzymatically active form by treatment of the membrane with papain [14]. These proteins are anchored in the membrane only by a short hydrophobic peptide chain [4], and are generally more easily removed from the membrane [15] than is alkaline phosphatase.

Addendum

After completion of the manuscript de Grip and Bovee-Geurts [16] also reported a modified synthesis of octylglucoside. This method involves column chromatography which is not needed in the method described above and the product has a specific rotation of -31.3° compared to -37.1° of the product synthetized by us. This might indicate a still higher purity of our product.

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