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An improved purification of lactose permease

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The integral membrane protein lactose permease of *Escherichia coli* was purified to homogeneity in detergent micelles. No other proteins could be detected in the final product. The molar ratio of lactose permease to lipid was less than 0.2 in detergent solution, thus the preparation was essentially lipid-free. All molecules were functionally active as shown by substrate binding.

Ion-exchange chromatography; Membrane protein; (*E. coli*)

1. INTRODUCTION

Lactose permease (LP) is an integral protein of the inner membrane of *Escherichia coli*. It catalyzes the cotransport of galactosides together with protons across the membrane (reviews [1,2]). The functional unit for transport is the monomer [3,4], which consists of 417 amino acids [5]. LP is composed of 12–14 membrane-spanning helices [6,7], 10 of which are hydrophobic or amphipathic and are probably arranged on a ring, thus protecting the hydrophilic core of the protein from the lipids [3,7].

The procedures to purify LP which have been published [8,9] start by preparing vesicles of the cytoplasmic membrane of the overproducing strain *E. coli* T206. About 15% of the proteins of these vesicles is LP [10]. Subsequently, these vesicles were solubilized in detergent and subjected to DEAE anion-exchange chromatography, where LP in contrast to most other proteins is not retained. These purification procedures result in LP which is more than 95% pure with respect to other proteins. However, no purification is achieved with respect to lipids and hydrophobic low-M_r impurities.

In a recent publication, Li and Tooth [11] reported the use of a CM52 cation-exchange column, in addition to the DEAE anion-exchange column. LP was bound to the CM52 column, and after washing the column with buffer, LP was eluted by stepwise addition of KCl. This improved method yields a lipid:LP ratio of 4. The preparation was suitable for the preparation of crystalline filaments of LP.

Here, an improved purification method is described, which lowers the lipid:LP ratio to about 0.2. No other proteins are detectable. Using this preparation, it may be possible to obtain better crystals which will permit higher resolution electron microscopy or X-ray diffraction analysis of LP.

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Abbreviations: LP, lactose permease; DodOMalt, dodecyl-*O*-β-D-maltoside; MalNEt, *N*-ethylmaleimide; DMPC, dimyristoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Np-α-Gal, *p*-nitrophenyl-α-D-galactoside; DEAE, diethylaminoethyl

2. MATERIALS AND METHODS

2.1. *Chemicals*

The ion-exchange materials Mono Q, Q-Sepharose Fast Flow and Sephadex A25 were from Pharmacia (Uppsala). [^{14}C]DMPC, [^{14}C]DPPE and MalN[^{3}H]Et were purchased from NEN (Dreieich). DodOMalt was from Calbiochem (Frankfurt). [^{3}H]Np-α-Gal was a kind gift from Dr P. Overath.

2.2. *Strains and preparation of membrane vesicles*

The permease-overproducing strain *E. coli* T206 and the

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permease-deficient strain *E. coli* T184 were grown as described [12]. Vesicles of the cytoplasmic membrane were prepared [12] and pre-extracted with 5'-sulfosalicylate and 25 mM Na cholate [3,7]. Such preparations from T206 contain 3–4 nmol LP/mg membrane protein [13].

In order to follow the yield of LP during purification, the cytoplasmic membranes were supplemented with 0.5 kBq MalN[^{3}H]Et-labeled carrier/mg protein [13]. To some preparations of the cytoplasmic membrane, 200 kBq/mg protein of [^{14}C]DMPC or [^{14}C]DPPE was added in order to follow the fate of the lipids.

2.3. *Mono Q chromatography*

Vesicles of the cytoplasmic membrane were diluted to a protein concentration of 5 mg/ml in 25 mM Tris-HCl buffer (pH 8.6), 1 mM NaEDTA, 50 mg/ml glycerol. The same volume of buffer containing 15 mg/ml DodOMalt was added whilst stirring on a vortex mixer [9]. The solution was sonicated for 5 min in a bath sonifier and centrifuged for 10 min at 10000 × *g*. 0.5 ml (1.25 mg protein) were applied to the strong anion exchanger Mono Q (HR 5/5, bed volume 1 ml), which had been equilibrated with the above buffer containing 1 mg/ml DodOMalt. The column was eluted by an NaCl gradient from 0.0 to 1.0 M in 25 mM Tris-HCl (pH 8.6), 1 mM NaEDTA, 50 mg/ml glycerol, 1 mg/ml DodOMalt. 55 fractions (1 ml) were collected at a rate of 0.3 ml/min. Elution of protein was detected by measuring the absorbance at 280 nm.

To prepare larger amounts of LP, the Mono Q column was replaced by Q Sepharose Fast Flow. For 10 ml solubilized cytoplasmic membranes (25 mg protein), 20 ml anion exchanger were used. The conditions for elution of LP were essentially the same as above, except that ~100 fractions (4 ml) were collected at a rate of 1 ml/min.

2.4. *Sephadex A25 chromatography*

Fractions enriched in LP were pooled and dialysed 3 times for at least 3 h each against 10 mM Tris-HCl (pH 8.0), 1 mM NaEDTA, 50 mg/ml glycerol, 1 mg/ml DodOMalt. The dialysate was subsequently applied to a Sephadex A25 column (DEAE anion exchange) which was equilibrated in the above buffer. The previously used DEAE anion-exchange material Ecteola 23 (Serva, Heidelberg) [9] may be replaced by Sephadex A25 (Wright, J.K., personal communication). For 1.25 mg protein applied to the Mono Q column, a 1 ml bed volume Sephadex A25 column was used. The column was eluted with the above buffer. Fractions of 0.5 ml were collected. LP appears in the effluent as detected by the absorbance at 280 nm and MalN[^{3}H]Et-labeled permease.

The solution was concentrated by ultrafiltration when necessary with Diaflo ultrafiltration membranes PM10 (Amicon, Douvers, MA). By one ultrafiltration step the volume was reduced by a factor of no more than 10 in order to prevent inactivation of LP by excessively high concentrations of DodOMalt. Therefore, each ultrafiltration step was followed by 18 h dialysis against the 10-fold volume of buffer without DM. Thus, the concentration of DodOMalt varies only between 1 and 10 mg/ml.

2.5. *Miscellaneous procedures*

SDS gel electrophoresis [14] was performed with the modifications described in [9]. Gels were stained with silver [15]. Protein concentrations were determined according to Peterson [16].

The number of binding sites of LP in DM micelles was determined by flow dialysis using the radiolabeled galactoside [^{3}H]Np-α-Gal [12,13] after concentration of the solution by ultrafiltration to an LP concentration of about 10 μM.

3. RESULTS

Fig.1 shows the elution profile of the Mono Q column. Cytoplasmic membranes of *E. coli* T206 were applied to the column, and after washing the column with 25 ml buffer, the bound proteins were eluted with an NaCl gradient. LP appeared in a sharp peak around fraction 30 at 100 mM NaCl. Obviously, the peak was not completely separated from small peaks between fractions 27 and 33, thus the purification using Mono Q solely was not completely satisfying with respect to other proteins. Applying pooled fractions 29–31 to the Sephadex A25 column resulted in a pure preparation of LP as shown by the silver-stained SDS polyacrylamide gel (fig.2). Lane 1 represents proteins of the cytoplasmic membrane, lane 2 pooled fractions 29–31 of the Mono Q run, and lane 3 the eluate of the Sephadex A25 column. As judged from SDS gel electrophoresis, LP runs with an apparent molecular mass of 31 kDa. After the Mono Q run, some minor contaminants of higher and lower molecular mass can be seen. In particular, one strong band of 29 kDa was not eliminated. No impurities were detectable after application of dialyzed fractions 29–31 to the Sephadex A25 column, as judged from lane 3 in fig.2. For comparison, lane 4 shows the same purification by Mono Q and Sephadex A25 columns using cytoplasmic membranes of the LP-deficient strain *E. coli* T184 as starting material. Lane 4 is devoid of proteins even after overloading the gel. These results show that there are no other proteins present which comigrate on the gel with the broad LP band.

The addition of ^{14}C-labeled lipids to the cytoplasmic membrane allows one to follow the purification of LP with respect to lipids. Both [^{14}C]DMPC and [^{14}C]DPPE were used in order to investigate the influence of the charge of the lipid head group. Fig.1 shows the elution of radioactivity from the Mono Q column on a logarithmic scale. The elution profile was identical for DMPC and DPPE. Both types of lipids appeared in the

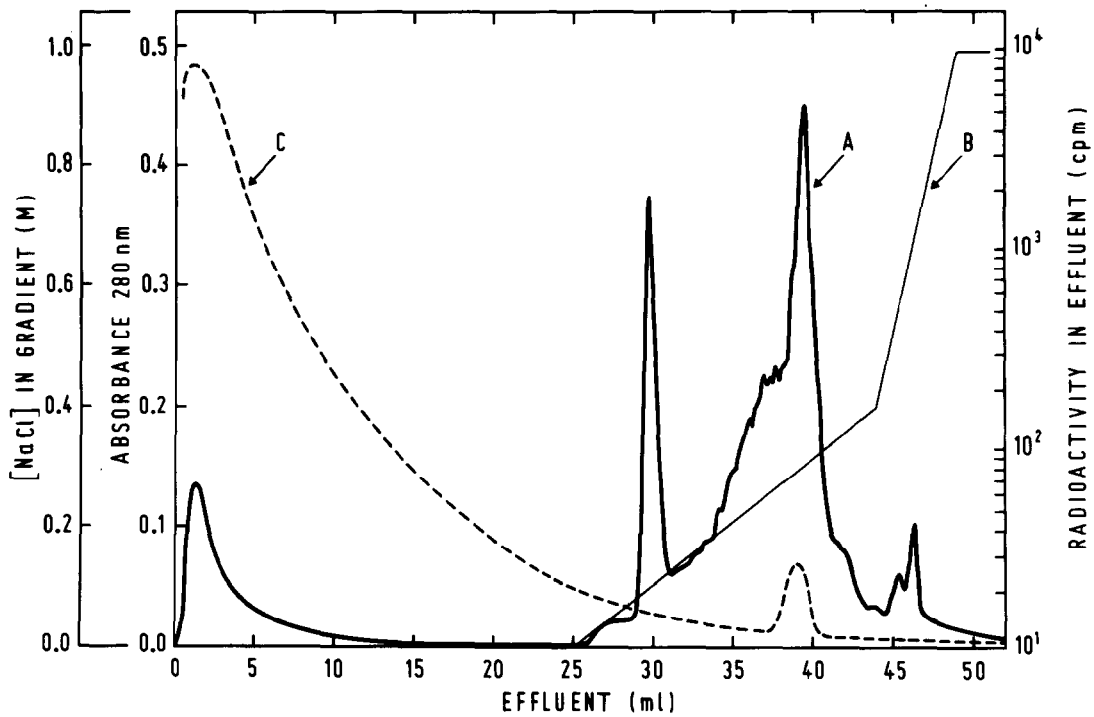

Fig.1. Chromatography of cytoplasmic membranes of *E. coli* T206 on the Mono Q column. (A) Elution profile of protein. Absorption of tryptophan was detected at 280 nm. (B) The column was eluted by an NaCl salt gradient. The curve indicates the concentration of NaCl during elution. (C) Effluent of ^{14}C-labeled lipids on a logarithmic scale. DMPC and DPPE showed the same elution profile.

flow through of the Mono Q column, although some tailing is observed. This tailing is presumably due to the slow exchange of lipids between the micelles which are bound to the column and those which flow through the column.

The yield of LP recovered following

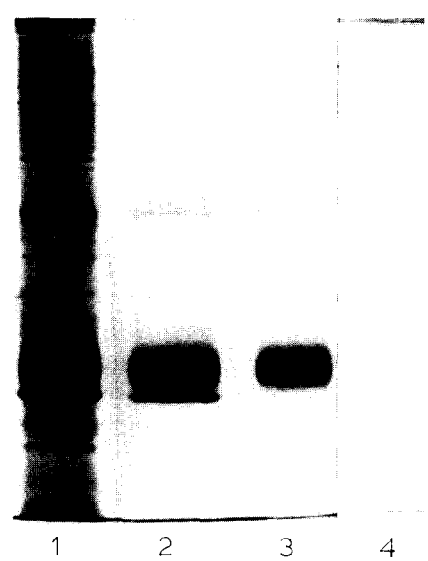

Fig.2. SDS gel electrophoresis of LP before and after purification on the Mono Q and Sephadex A25 columns. The gel was stained with silver using the method of Heukeshoven and Dernick [15]. Lanes 1–3 contain about the same amount of LP, as judged from MalN[^{3}H]Et-labeled LP before applying the samples to the gel. Lane 4 was loaded with an amount of sample corresponding to that of lane 3. Lanes: (1) Cytoplasmic membranes of the LP-overproducing strain *E. coli* T206 pre-extracted with 5′-sulfosalicylate and Na cholate. The LP concentration in this preparation was 4.0 nmol/mg protein. 25 μg protein were applied to this lane. (2) Pooled fractions 31–33 of the elution of the Mono Q column (cf. fig.1). (3) Pooled fractions 31–33 of the Mono Q column (lane 2) were applied to the Sephadex A25 column. This subsequent purification step results in a homogeneous protein which migrates as single band of apparent molecular mass of 31 kDa, which is typical of monomeric LP in SDS micelles. (4) Cytoplasmic membranes of the LP-deficient strain *E. coli* T184 were purified by Mono Q and Sephadex A25 chromatography according to the protocol for strain T206 membranes, i.e. by Mono Q and Sephadex A25 chromatography.

chromatography on the Mono Q column was 12%, as estimated from the recovery of MalN[^{3}H]Et-labeled LP. After Sephadex A25 chromatography, the yield of LP decreased to 8.5%. After concentrating the eluent, determination of the number of binding sites by flow dialysis revealed a recovery of 8.7%.

From the yields of LP and of ^{14}C-labeled lipids the molar ratio of lipid:LP can be calculated. If a lipid:LP molar ratio of 80 is assumed for the cytoplasmic membrane of *E. coli* T206 [11], the lipid:LP ratio after Mono Q and Sephadex A25 chromatography was found to be less than 0.2.

4. DISCUSSION

The purification method described here results in a homogeneous preparation of LP. Even on overloaded silver-stained SDS gels, other proteins were not detectable. The molar ratio of LP:lipid is less than 0.2. Although the yield of LP is low, this method is an improvement not only with respect to the use of a single DEAE anion-exchange column [8,9], but also regarding the Ecteola/CM-52 method of Li and Tooth [11], as the lipid:LP ratio is lowered by a factor of more than 20. A further advantage is that LP may be eluted from the Mono Q column by a linear NaCl gradient in a single sharp peak, in contrast to the Ecteola/CM-52 purification [11] in which LP is recovered in a broad peak. In our preparation, no other protein impurities and no aggregated LP could be observed. This is also in contrast to the method of Li and Tooth, where higher concentrations of KCl used for elution of LP from the CM-52 column also lead to simultaneous elution of at least three other proteins as well as dimers of LP. However, it is still unclear why LP migrates in such a broad band on SDS gels (fig.2 this study, and [8,9,11]).

Comparisons of the yields of LP determined by the quantification of radiolabeled LP, and by a number of binding sites, shows that essentially all LP molecules are functionally active. By means of MalN[^{3}H]Et labeling, the total number of polypeptide chains is determined, irrespective of whether the protein is active or denatured. The yield evaluated thereby was 8.5%, which is almost identical to that of 8.7% determined by flow dialysis which measures only the number of functionally active LP molecules.

An important feature concerning the function of LP is the LP:lipid ratio of 0.2. Although not all types of lipids were tested systematically in this study, this ratio shows that there is no specific association of LP and lipids, at least not with phosphatidylcholines and ethanolamines, of which the latter accounts for 75–80% of the phospholipid head groups of *E. coli* [17].

The Mono Q purification step unfortunately results in a quite low yield of only 12–13%. Variations of buffer and elution conditions have not yet enhanced the yield. However, as the LP-overproducing strain *E. coli* T206 is used, which grows easily in a simple medium, it is nevertheless possible to obtain milligrams of highly purified LP within some days. Therefore, this purification method provides a suitable material for experiments which depends on highly purified LP. It seems possible that the quality of LP filaments [11] can be improved or that even three-dimensional crystals of LP may be obtained.

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