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AN ENRICHMENT PROCEDURE FOR A HIGHLY ACTIVE T5-RECEPTOR PREPARATION BY DEAE-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY IN TRITON X-100 CONTAINING SOLUTION AT ELEVATED TEMPERATURES

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SUMMARY

This paper describes the purification of a minor outer membrane protein from *Escherichia coli* B by repeated DEAE-cellulose ion-exchange chromatography at 40°C in phosphate buffer containing 2% Triton X-100. A 480-fold enrichment relative to a crude preparation of membrane fragments was obtained. The preparation is highly active as a receptor for the bacteriophage T5. The active particles are homogeneous in size with a molecular weight of 230,000 daltons.

INTRODUCTION

The outer membrane of *Escherichia coli* is a highly specialized and rigid bilayer membrane. It enables the Gram-negative bacterium to survive in the bile detergent-containing environment of the gut. Owing to their easy extractability, the major outer membrane proteins are very well characterized according to their function, structure and synthesis. Most of the minor outer membrane proteins, however, which provide many of the highly specific receptor sites for certain nutrients and the binding of bacteriophages and colicins[1–3], resist complete purification. Thus many investigations on their receptor functions were performed with rather crude extracts of the outer membrane or even with large membrane fragments[4–6].

The receptor protein for the bacteriophage T5 has been identified as the tonA (= fhuA) gene product[7]. It also serves as a receptor for the phages T1 and Phi80, for colicin M and it mediates the ferrichrome-promoted iron uptake. Its molecular weight is 78,000 daltons.

The initial *in vitro* interaction between the bacteriophage T5 and a TonA protein-containing membrane particle of nearly 50 million daltons has been described by us as a bimolecular multi-step reaction, reversible in the first step and irreversible in the second[5,6]. The membrane particle under investigation consisted of protein,

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phospholipid and lipopolysaccharide (LPS) in approximately the same ratio as the complete outer membrane (mass ratio 1.00:0.79:0.48). Therefore it is not clear whether all reaction steps were caused by the TonA. A reversible interaction between the O-antigen of *E. coli* F-lipopolysaccharide and the bacteriophage T5 has been described[8]. To clarify this question we needed an almost pure TonA preparation. The simplest way to obtain pure TonA protein is to excise its band from sodium dodecylsulphate (SDS) polyacrylamide electrophoresis gels, elute the protein, and renature it in an appropriate buffer[9]. However, as the renaturation is not very effective this material is not suitable for kinetic experiments. Braun *et al.*[7] used an analytical DEAE-cellulose ion-exchange chromatography at room temperature for the identification of the receptor protein. In their preparation only a small fraction of the TonA protein had been solubilized.

The present paper describes a procedure for the enrichment of the TonA protein that provides a highly active receptor preparation with homogeneous size of the active particles by using repeated DEAE-cellulose ion-exchange chromatography at elevated temperatures. The kinetic and thermodynamic analysis of the reaction of these particles with T5 bacteriophages will be published in a later paper.

EXPERIMENTAL

Column chromatography

For gel filtrations the BioGels A-1.5m and A-50m (BioRad Labs., Munich, F.R.G.), and Ultrogel AcA34 (LKB, Munich, F.R.G.) were used. Ion-exchange chromatography was performed on Whatman DE52 DEAE-cellulose (c/o Vetter, Wiesloch, F.R.G.). The columns were first packed in Triton X-100-free eluent buffer in accordance with the prescribed packing procedure. For chromatography in Triton X-100-containing buffers, the columns were used after elution at the operational flow-rate and temperature with three bed volumes of eluent containing Triton X-100. The Triton X-100 (Packard) was used without further purification. The samples were preincubated for at least an hour at the chromatographic temperature before being applied to the columns.

Preparation of Weidel receptor

The crude receptor was isolated by extraction of *E. coli* B cells with sodium hydroxide and purified by differential centrifugation and gel chromatography on BioGel A-50m as described previously[6,7,10]. from 300 g of *E. coli* B we obtained 350 mg of this preparation.

Protein determination

Protein contents were measured by the Fluram method[11] using bovine serum albumin standards (Serva, Heidelberg, F.R.G.).

Discontinuous SDS polyacrylamide gel electrophoresis (PAGE)

Disc SDS-PAGE was performed as described elsewhere[12]. The gels were stained with Coomassie brillant blue.

Determination of receptor activity

On the assumption that the reaction between phages and receptor particles can, at least in the early phase, be described as a reaction of pseudo-first order, the receptor activity of the chromatographic fractions was determined kinetically. The surviving fraction of 1500 T5-st phages after 1 h incubation with a receptor sample in 0.5 ml of 10 mmol/l Tris-HCl pH 7.8 with 10 mmol/l sodium chloride and 0.3% Triton X-100 was estimated by plating as described previously[6]. The negative value of the natural logarithm of this fraction divided by the reaction time is defined as the kinetic receptor activity. This divided by the protein concentration yields the kinetic specific activity used in Table I.

TABLE I

PURIFICATION TABLE

Crude extract: sodium hydroxide extract of bacteria after differential centrifugation as described in ref. 10; Weidel receptor: this extract after purification on BioGel A-50m; the four DEAE-cellulose purifications show data relating to the chromatograms of Fig. 3.

	Protein (mg)	*Total activity (ml/h)*	*Specific activity (ml/h/mg)*	*Yield (%)*	*Enrichment*
Crude extract	3300	119790	36.3	100.0	1.0
Weidel receptor	350	58380	166.8	48.7	4.6
(1) DEAE-cellulose	156	57127	366.2	47.7	10.1
(2) DEAE-cellulose	28.4	49225	1733.3	41.1	47.8
(3) DEAE-cellulose	13.7	33135	2418.6	27.7	66.7
(4) DEAE-cellulose	0.87	15225	17500	12.7	482.0

RESULTS

Weidel receptor particles prepared from the outer membrane of *E. coli* B can be split by incubation in the presence of Triton X-100. Increased temperature improves the splitting. The quality of the splitting is shown by gel chromatography on a calibrated BioGel A-1.5m column (Fig. 1). Without Triton, all particles were eluted above the exclusion limit of $1.5 \cdot 10^6$ daltons (Fig. 1a) even at 40°C. In the presence of Triton a partial splitting was observed at 15°C (Fig. 1b).

Under conditions where complete splitting occurs, we would expect either that the integral membrane proteins are incorporated into Triton micelles or that they have the hydrophobic part of their surface covered with Triton molecules. The molecular weight of a Triton micelle under the given conditions is *ca.* 100,000 daltons[13]. Therefore the largest particles obtained should be no larger than 300,000 daltons. Almost complete splitting was achieved by preincubating the sample for 1 h at 40°C in the column buffer before application to a column with the same temperature (Fig. 1c). This was not only a kinetic effect of the Triton-caused splitting. Longer incubation times at 15°C gave no better results than Fig. 1b (data not shown). Fig. 1d shows that the effect was reversible, at least in part. The sample preincubated at 40°C and applied to the 15°C column showed an elution pattern similar to that in Fig. 1b.

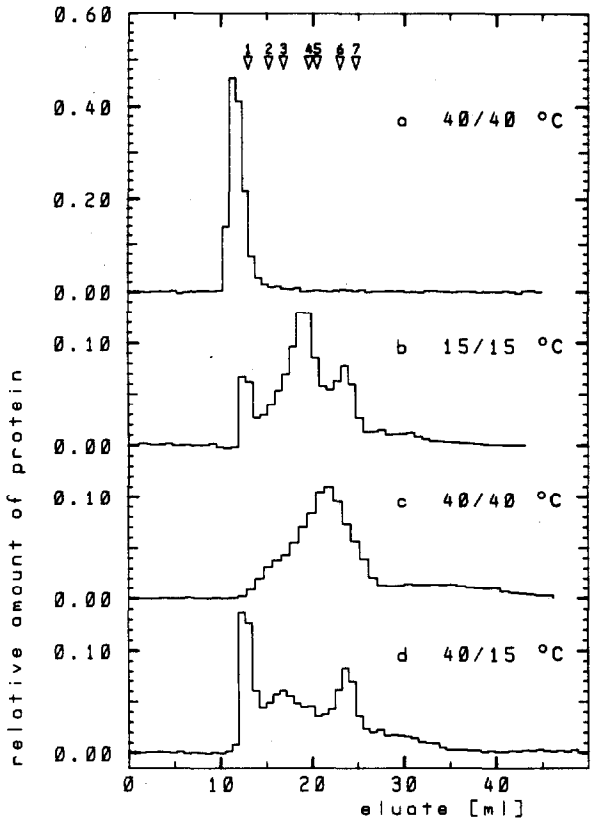

Fig. 1. Analytical BioGel A-1.5m gel filtration of crude T5 receptor, purified by differential centrifugation and gel filtration on BioGel A-50m. The two temperatures are those of the preincubation and the column. After preincubation for 1 h at the appropriate temperature, a 0.05-ml sample containing 0.032 mg of ^{14}C-labeled receptor protein was applied to a calibrated column (58 × 0.9 cm I.D.) and eluted at 2 ml/h. Except for (a), 0.3% Triton X-100 was present in the column buffer (10 mmol/l Tris–HCl pH 7.8, 50 mmol/l sodium chloride, 0.02% sodium azide). The radioactivity in the eluate was estimated. The areas under the curves are normalized to 1.0. Calibrating substances: 1 = main peak of dextran blue; 2 = thyroglobulin (669,000 daltons); 3 = ferritin (440,000 daltons); 4 = catalase (232,000 daltons); 5 = lactate dehydrogenase (140,000 daltons); 6 = bovine serum albumin (67,000 daltons); 7 = ovalbumin (43,000 daltons).

Encouraged by these results and by the high stability of the receptor activity at 40°C which had been found previously[6], we decided to use ion exchange chromatography at 40°C in Triton X-100-containing buffer for the enrichment of the TonA protein.

When working with solutions of non-ionic detergents at elevated temperatures and increasing ionic strength, it must be remembered that severe changes in the micellar structure occur at the cloud point of the detergent which should be avoided for reproducible experimental conditions[14]. The cloud points for 10 mmol/l phosphate buffer (pH 7.0), with 0.02% sodium azide and 2% Triton X-100 are 61°C without, 56°C with 0.5 mol/l, and 49°C with 1 mol/l sodium chloride (Fig. 2). Braun *et al.*[7] have shown that a large amount of inactive material is already separated at

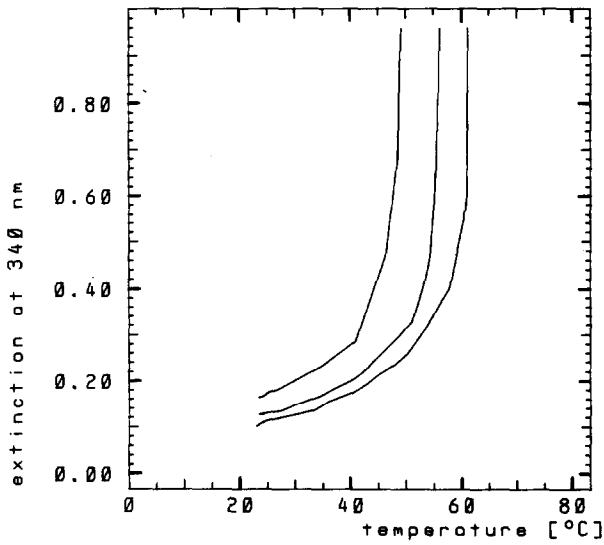

Fig. 2. Estimation of the cloud point of 2% Triton X-100 in 10 mmol/l phosphate buffer (pH 7.0) with increasing amounts of sodium chloride (0.0, 0.5, and 1.0 mol/l from right to left). The cuvette with 1-ml sample at ambient temperature was put into the cuvette housing of a Zeiss PMQ2 photometer at 90°C. During the equalization of the temperature, the increased light scattering was measured as the increase in the extinction at 340 nm, and the temperature in the cuvette was simultaneously measured with a Braun Tastotherm P60 thermometer. The temperature increased at a rate of *ca.* 0.5°C/min.

room temperature. Therefore we started our purification of TonA protein with ion exchange chromatography in 10 mmol/l phosphate buffer (pH 7.0), supplemented with 0.05% sodium azide and 2% Triton X-100 at 20°C (Fig. 3a). In order to achieve better solubilization of the membrane fragments, the active fractions of the first run were dialysed against salt-free buffer and then rechromatographed on the same column at 40°C. The higher resolution is obvious (Fig. 3b). The two subsequent runs at 40°C (Figs. 3c and d) showed further remarkable increases of the specific receptor activity. As a result we achieved an overall 480-fold purification of the TonA protein relative to the crude Weidel extract. The material that was eluted immediately after starting the salt gradient in Fig. 3c and d was pure OmpF protein (identified by SDS–PAGE). Purification is summarized in Table I.

The SDS–PAGE pattern of the enriched TonA preparation, compared with the complete outer membrane, prepared according to Osborn *et al.*[15], and Weidel's extract, is given in Fig. 4. Planimetry of a densitometer scan of gel c yielded a TonA content of 19%. The active particles in this preparation have a well defined molecular weight, which can be estimated on a calibrated Ultrogel AcA 34 column to be 230,000 daltons, corresponding to a Stokes radius of 4.6 nm (Fig. 5). The exact number of receptor particles per milligram of protein can be calculated when a slight numerical excess of phage particles is incubated with receptor particles and the number of surviving phages is measured after the reaction is completed (for a detailed description of the method see ref. 6). Our preparation had $4.48 \cdot 10^{14}$ receptor particles per milligram of protein.

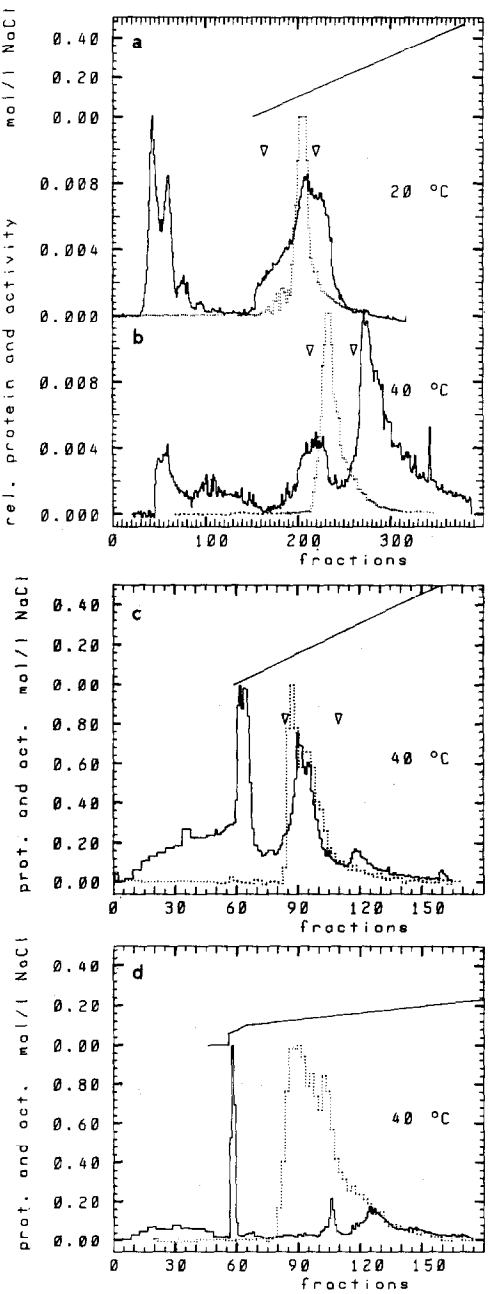

Fig. 3. DEAE-cellulose ion-exchange chromatographic purification of T5-receptor. Columns: (a) and (b), 55 × 3 cm I.D.; (c) and (d), 20 × 1.6 cm I.D. Buffer: 10 mmol/l phosphate buffer (pH 7.0), with 2% Triton X-100 and 0.05% sodium azide; the inserts show the sodium chloride gradients). In (a) and (b) 5-ml fractions and in (c) and (d) 2.5-ml fractions were collected. The relative amount of protein (Fluram method) in the eluate is given by the solid lines, the relative kinetic activity is given by dotted lines. The active fractions (between the two arrows) were applied in the subsequent run after exhaustive dialysis against salt-free buffer.

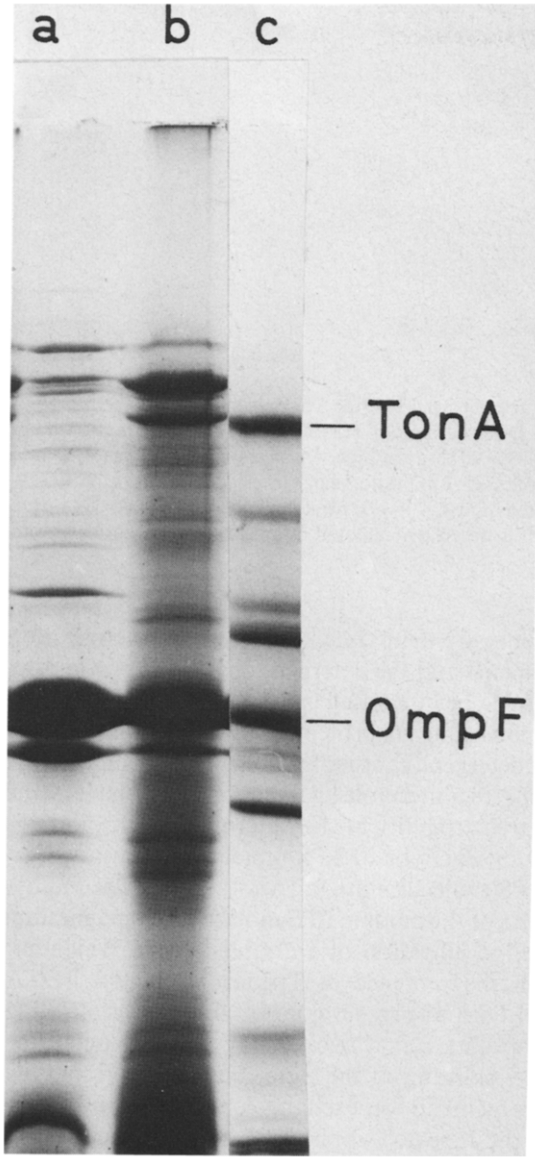

Fig. 4. (a) SDS–PAGE of outer membrane of *E. coli*, prepared according to Osborn[15]. (b) Crude T5-receptor after differential centrifugation and BioGel A-50m-purification (c) Preparation obtained from the ion-exchange chromatography of Fig. 3d.

DISCUSSION

Non-ionic detergents such as Triton X-100 are frequently used for the solubilization of biological membranes. At detergent concentrations below the critical micelle concentration (cmc), the detergent is at first integrated into the membrane. Higher detergent concentrations will split the membranes into smaller fragments.

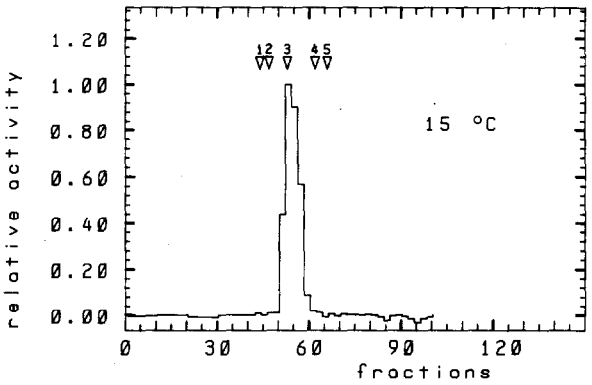

Fig. 5. Molecular weight determination of the active particles in the TonA preparation by gel filtration on Ultrogel AcA 34. Column, 55 × 0.9 cm I.D. in 10 mmol/l Tris–HCl (pH 7.8), 10 mmol/l sodium chloride, 0.02% sodium azide, 0.3% Triton X-100; 0.0012 mg of protein was applied; elution rate, 2 ml/h; temperature, 15°C; fractions of 0.4 ml were collected. Calibration substances: 1 = dextran blue; 2 = ferritin (440,000 daltons); 3 = catalase (232,000 daltons); 4 = bovine serum albumin (67,000 daltons); 5 = ovalbumin (43,000 daltons). The ordinate gives the relative amount of kinetic activity, normalized to a maximum of 1.0.

Above the cmc the membrane fragments are delipidated according to the partition equilibrium of lipids between the fragments and the detergent micelles. Appropriately, the amphiphilic membrane proteins can be integrated into detergent micelles[16–18]. This is true for normal membranes, consisting of proteins and phospholipids. The grade of the splitting depends on the detergent chosen, the buffer, the temperature, and above all on the strength of the specific intermolecular interactions between the components of the membrane. The extreme rigidity of the outer membrane of gram-negative bacteria is mainly due to the interactions of LPS molecules with the membrane proteins and to those of the LPS molecules among another. Besides the hydrophobic interactions, the cross-linking of the anionic LPS molecules by magnesium ions seems to be important. However, gel filtration of a double-labeled Weidel receptor ([^{3}H]LPS and [^{14}C]protein) in the presence of Triton X-100 and EDTA showed no increase of separation of LPS and protein compared with gel filtration without EDTA (at room temperature; data not shown). Only an increase in the temperature resulted in an appropriate splitting of the outer membrane of *E. coli*, which enabled the separation of the proteins in ion-exchange chromatography.

The increase in enrichment by the repeated application of the same method was remarkable. This increase seems to be based on the special properties of a detergent-containing separation system above the cmc. Ion-exchange separation is involved, as well as a phase partition equilibrium. Two effects are important. First, in the chromatograms presented in Figs. 3b–d the samples were applied highly diluted as they were obtained from the preceding runs after dialysis against salt-free column buffer. Dilution shifts the phase equilibrium to better splitting of the membrane components. Second, as the buffer was the same in all runs but decreasing amounts of protein were used, the mass ratio of detergent to protein increased from 2.5 to 40 mg of Triton X-100 per milligram of protein. This should also improve the splitting.

In the final preparation the active particles had a molecular weight of *ca.*

230,000 daltons. The number of TonA molecules necessary for one receptor site can be calculated from the number of receptor particles per milligram of protein and the TonA-content in our preparation. When the reciprocal of the number of receptor particles per milligram is multiplied by Avogadro's number and the relative amount of TonA protein (0.19), and divided by the molecular weight of TonA (78,000 daltons), the number of 3.3 TonA molecules per receptor particle is obtained. Considering all the possible inaccuracies this is of course a rather rough estimate, and the obvious conclusion that three TonA molecules form a receptor site of 234,000 daltons cannot be drawn without further information. The results presented in this paper might, however, be interpreted as a first hint in this direction. At present, other interpretations must be kept in mind which consider the possible incorporation of proteins into Triton micelles, the partial loss of receptor activity of a fraction of the TonA molecules, or other compositions of the active particles with more or less Triton bound. The purification method used might be helpful in other cases where minor components of membrane fragments are to be isolated.

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