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Use of n-Octyl-b-D-thioglucopyranoside in the isolation of a bacterial membrane protein: an improved method for immunological applications

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Abstract — Surfactants are essential in the isolation of integral membrane biomolecules from biological membranes of different microorganisms. Our objective is the isolation of the major outer membrane protein (MOMP) of C*hlamydia trachomatis*, to use it in the preferential and specific detection of immunoglobulins. We have improved a method using an alkylglycoside surfactant, *n-Octyl*-b-*D-thioglucoside* (OTG), which is a non-ionic detergent used in membrane solubilization due to its "weak" action preserving the biological and functional properties of solubilized biomolecules. Different solubilization conditions, such as surfactant and salt concentrations, temperature and the presence of additive (di-thiothreitol (DTT)), were tested. To know the influence of the parameters previously indicated on the micellar properties and the solubilizing ability of the surfactant, we have studied the micellization process of OTG under several conditions by using static fluorescence measurements. MOMP was isolated by a simplified method consisting of a two-step extraction using OTG and DTT with an optimization of the experimental conditions. In this way, MOMP turns out to be separated from other biomolecules (i.e., lipopolysaccharides) in order to avoid immunological cross-reactions among the different chlamydial species. Furthermore, gel electrophoresis experiments showed monomeric MOMP without multi-aggregation, even after removing DTT and OTG molecules by dialysis of the OTG-DTT soluble fraction. The isolated protein (MOMP) is used to coat microplate wells to develop immunoenzymatic assays (ELISA). The preliminary immunological study shows the detection of specific IgG of *Chlamydia trachomatis,* with adequate values of the immunological parameters : sensibility and specificity. Moreover, these results show the role of surfactant molecules in the

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binding process of proteins to solid-liquid interface.

Keywords : *n-octyl-b-D-thioglucopyranoside, bacterial membrane protein, immunological application, solubilization, immumoenzymatic assays.*

INTRODUCTION

The outer membrane (OM) of elementary bodies (EBs) of *Chlamydia trachomatis* contain several proteins and other biomolecules, including lipopolysaccharides (LPS), with antigenic character. The 40 kDa major outer membrane protein (MOMP) appears to be a good candidate for preferential and specific detection of IgG. This immunodominant protein is both, a target of neutralizing antibodies and the serotyping major antigenic determinant, and therefore, it has been a focus of diagnostic and experimental investigation [1, 2]. MOMP is a multifunctional protein, and besides the antigenic role in the infectious process, it takes part in the maintenance of the structural rigidity, via disulphide bond cross-linking within the EB outer membrane. Thus, MOMP molecules are found in the form of large hetero-oligomers imbibed in a hydrophobic environment [3, 4].

The importance of MOMP molecules in serological immunoreactions was shown by Bas et al. comparing different serological reactive tests [5]. The best sensitivity and specificity was obtained with IgG responses to MOMP, but cross-reactions have been reported as a consequence of genus antigen of lipopolysaccharide character [5]. Members of the Chlamydiaceae family are gram-negative bacteria containing LPS in the outer membrane, which is a well characterized genus-specific antigen as well as several proteins, species-specific antigens, which may be closely associated, but not covalently bounded, to the LPS [6-8].

On the other hand, surfactants are indispensable in solubilizing processes used for the extraction, isolation and purification of biological macromolecules. From this point of view, surfactants classified into ionic and non-ionic systems present a very different behaviour. Ionic surfactants, i.e., sodium dodecyl sulphate (SDS), are efficient solubilizers although they also denature [9]. However, non-ionic surfactants are generally “mild” and it is assumed that they solubilize membrane proteins without affecting important structural features.

Accordingly, MOMP of *C. trachomatis* could be solubilized by ionic surfactants, i.e., SDS, with the loss of biological functionality as a consequence of protein denaturation. When “mild” surfactants (non-ionic surfactants) are used MOMP is not solubilized in the absence of a reducing agent [10]. Thus, alkylglucoside surfactants have been used together with reducing agents to solubilize MOMP from chlamydial bacteria [10, 11]. These are non toxic, non-irritant and biodegradable non-

ionic surfactants, and have been increasingly used in the solubilization, reconstitution and crystallization of membrane proteins with the retention of functional properties [12-14]. Octyl-b-D-glucopyranoside (OTG) is widely used in biomembrane research and reconstitutions processes of biological membranes [15, 16]. In this way, its sulphur variant, octyl-b-D-thioglucopyranoside (OTG) shows a similar power of solubilization with a higher stability and lower cost [17].

Another important aspect related to the selection of the surfactant system, is its elimination from the soluble fraction. Due to the relatively high critical micelle concentration (CMC) of OTG, the aqueous solution of OTG-protein complexes would be amenable to dialysis in order to elute the surfactant molecules from the protein solution [13, 17]. This is an important consideration taking into account the interaction between the surfactant and the solid-liquid interfaces, which could cause a significant interference in the interaction of protein molecules and polymeric interfaces [18, 19]. In the light of this, it may be relevant to study the presence of other molecules in the protein solution along with other possible structural changes in the protein molecules, i.e., molecular aggregation, because the purity and structural conditions of the antigenic molecules are decisive in order to get a satisfactory interaction with polymeric systems and to develop immunodiagnostic test for the correct detection of specific antibodies [20, 21].

In our investigation, we use a simplification of Bavoil's procedure [10] with only one surfactant (OTG) and two steps. We establish the most favourable conditions that allow the extraction and isolation of MOMP of *Chlamydia trachomatis* with an adequate yield, having a protein solution in optimal conditions of purity (absence of other membrane biomolecules), structure (without molecular aggregation) and functionality (adequate immunological behaviour). By means of the fluorescence technique and dialysis experiments, we have studied the interactions, membrane-surfactant, protein-surfactant, and the removing of surfactant molecules from the soluble fractions. Accordingly, we obtain information about the purity and structure of MOMP molecules by gel electrophoresis in different situations. Finally, the isolated protein was used for covering microwells of titration plates in order to make a preliminary immunological evaluation with positive and negative serum samples of *Chlamydia trachomatis*, previously tested with the reference serological technique: indirect immunofluorescence. Relevant conclusions are obtained by comparing the optical response with those obtained by using a commercially available ELISA test (Vircell, SL).

EXPERIMENTAL

Materials : All chemicals used in our study were of analytical grade and were used

without further purification. The surfactant (OTG), the reducing agent (DTT) and the fluorescence probe (pyrene) were purchased from Sigma. All experiments were performed with freshly prepared solutions and doubly distilled water was used throughout the present study.

Methods : Fluorescence measurements were recorded on a SPEX FluoroMax-2 steady-state spectrofluorimeter in the "S" mode with band-passes for excitation and emission of 1.05 nm. All fluorescence measurements were made at 25.0 ± 0.1°C. Fluorescence emission spectra of a number of surfactant solutions containing 1-2 mM of pyrene were recorded by using an excitation wavelength of 335 nm, and the intensities I_1 and I_3 were measured at the wavelengths corresponding to the first and third vibronic band located near 373 and 384 nm. The ratio I_1/I_3 is the so-called pyrene 1 : 3 intensity ratio.

Chlamydia trachomatis (LGV II strain 434, ATCC VR-902B, preserved under liquid nitrogen for long period storage) was grown onto McCoy cell monolayers in 150 cm^2 flasks with MEM medium supplemented with 1 μg/ml cycloheximide, 5.4 mg/ml glucose and 10% FBS. After 48 hours, 100% infected monolayers were scrapped onto the medium with the help of sterile glass beads. Cells were sonicated (Bandelin Sonopuls, Germany) at 30W for 30 s in an ice bath. EBs were obtained by differential centrifugation: a first low speed step (800 rpm, 5 min, no brake) was used to remove cell debris. EBs were pelleted from the supernatant and washed thrice with PBS at 39000g for 30 min. A final low speed centrifugation was carried out in a microfuge (1000 rpm, 5 min). Purity and absence of cell debris were assessed by microscopy.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done by using the Laemmli method [22] with 12.5 % acrylamide gels. The protein bands were then visualized by the Coomassie blue stain method. Lipopolysaccharide molecules in Polyacrylamide gels were detected by using a silver stain based on a LPS oxidation method [23].

Protein spots from gel samples were excised manually and then processed automatically using a Proteineer DP protein digestion station (Bruker Daltonics, Bremen, Germany) [24]. Next, a MALDI peptide mass fingerprinting and database search was developed. Thus, a 0.3 µl aliquot of matrix solution (5 g/l 2.5-dihydroxybenzoic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid) was manually deposited onto a 400 : m AnchorChip™ MALDI probe and allowed to dry at room temperature. Then 0.3 : l of the above extraction solution were added and again allowed to dry at room temperature. Samples were measured in a Bruker Reflex™ IV MALDI-TOF mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT™ source in the positive ion reflector mode using delayed extraction. The ion acceleration voltage was 20 kV. The measured

tryptic peptide masses were transferred through MS BioTools™ program as inputs to search the NCBInr database using Mascot™ software (Matrix Science, London, UK). No restrictions were placed on the species of origin of the protein and the allowed protein molecular mass was 1-200 kDa. Up to one missed tryptic cleavage was considered and a mass accuracy of 70 ppm was used for all tryptic-mass searches.

The immunofluorescence assay was carried out with a commercial kit (Vircell SL, Spain) based on the microimmunofluorescence technique, which employs simultaneously elementary bodies of *C. trachomatis*, *C. pneumoniae* and *C. psittaci*.

For the ELISA technique, plates (Maxisorb Nunc, Denmark) were coated with purified MOMP diluted in phosphate-buffered saline and incubated overnight at room temperature. Plates were blocked with calf serum and used for assaying human sera. Samples were tested diluted at 1/20 in a buffer containing calf serum and Tween 20 for 45 min. Peroxidase labelled conjugate (antihuman IgG, Fc specific) was incubated for 30 min. The reaction was initiated with a TMB/H_2O_2 substrate solution (Neogen, USA) and read at 450/620 nm after stopping it with 0.5M sulfuric acid.

Isolation of MOMP was done by a two step solubilization process. The purified EBs of *C. trachomatis* resuspended in PBS were incubated in the first step with different OTG concentrations ranging from below the CMC to several times the CMC at 37°C for 60 min and centrifuged at 40000 g for 20 min. The insoluble OTG fractions were resuspended in PBS and treated in the second step with the previously specified OTG concentration and 10 mM of DTT at 37°C, for 60 min. Different amounts of sodium chloride were added during this step to modify the ionic strength. After centrifugation, the insoluble fractions were resuspended in PBS under the same conditions of the first step. All samples, insoluble and soluble fractions, were aliquoted and stored at –80°C.

RESULTS AND DISCUSSION

The micellization of surfactants and their CMC are influenced by different experimental parameters and, therefore, the membrane solubilization process could also be affected. It is commonly accepted, as a general rule, that a decrease in the CMC value should increase the solubilization ability of surfactants [25]. Thus, in order to check the effect of some of these experimental parameters of the solubilization experiments on the micellar behaviour of OTG, we have examined the variations in the CMC of the surfactant at the experimental temperature, 37° C, with the addition of electrolyte (NaCl) and other important additive (DTT). As stated in the experimental section, CMC values were obtained by the well-established pyrene 1:3 intensity ratio (I_1/I_3) method [26-28]. I_1/I_3 ratio indices were recorded as a function of the total surfactant concentration in three different conditions: in the absence of

additives, in the presence of DTT (10 mM) and, finally, in the presence of DTT (10 mM) and NaCl (250 mM). Below the CMC, the I_1/I_3 ratio value corresponds to a polar environment. As the surfactant concentration increases the I_1/I_3 ratio decreases rapidly, indicating that pyrene is preferentially solubilized in the micellar phase, where the probe senses a more hydrophobic environment. Above the CMC, the I_1/I_3 ratio reaches a roughly constant value because of the incorporation of the probe into the micellar palisade layer [27]. From these plots, the CMC values were obtained as previously described [28], that is, the experimental data were fitted to a Boltzmann sigmoid type curve and the centre of the sigmoid was identified as the CMC value.

The results obtained from these experiments, Fig. 1, show a similar behaviour in all cases. We found 8.7 mM as the CMC of OTG in pure water, in good agreement with literature values [17]. This CMC value slightly reduces, 8.3 mM, in the presence of 10 mM of the reducing agent, DDT. This is a similar effect to the one obtained with other shorter-chain alcohols; it would mean that the presence

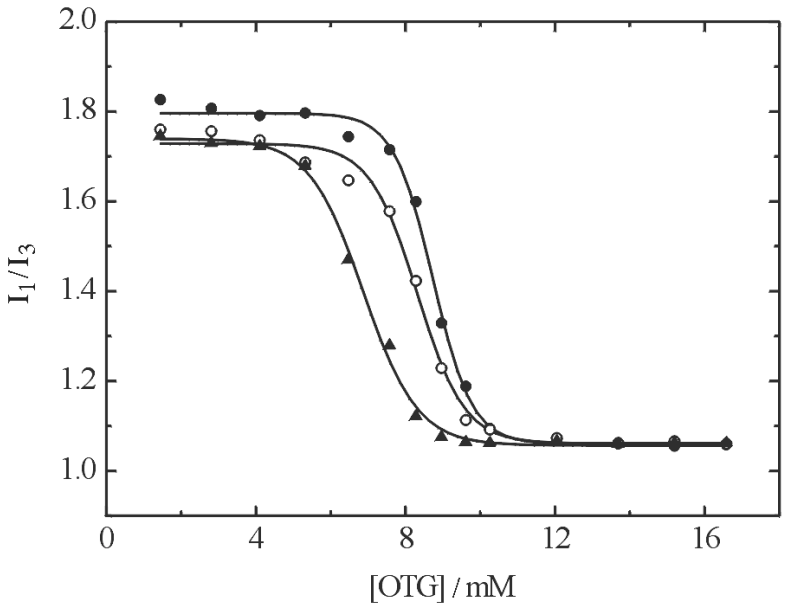

Fig. 1. Variation of the pyrene 1 : 3 vibronic peaks ratio (I_1/I_3) index, showing the change in the local polarity sensed by the probe, as a function of the surfactant concentration in different media, at 25°C : (•) water, (○) DTT (10 mM), and (▲) DTT (10 mM) + NaCl (250 mM). Solid lines are the best fit to a Boltzmann sigmoid curve.

of DTT in the solubilization process only affects disulfide bonds of the membrane protein skeleton without any direct effect on the solubilization ability of the surfactant.

On the other hand, an increase in the electrolyte concentration produces an additional reduction of the CMC value, 6.9 mM when [NaCl] = 250 mM. The most generally accepted explanation for this behaviour is given in the context of the "salting out" effect [29]. In this situation, the effects of surfactant on the bacterial membrane must be magnified as the ionic strength of the solution increases.

Another interesting aspect in Fig. 1 refers to the fact that the three curves collapse in the post-micellar region, where the pyrene 1 : 3 intensity ratio index takes roughly the same value in all the cases. This means that the polarity of the palisade layer sensed by the probe is not affected by the presence of the additives, suggesting that the interaction between these and OTG micelles takes place exclusively at the micellar interface.

An enhancement of the non-ionic surface activity with increased salt concentration due to the "salting out" effect can be expected [30]. The presence of electrolyte favours the hydration of ions, which means the salting out of hydrophobic groups of the detergent molecules and hydrophobic regions of the protein molecule. This situation allows an easier and closer interaction between surfactant and protein when OTG binds to hydrophobic regions on the native protein molecules to form mixed micelles or protein-surfactant complexes. Although non-ionic surfactant usually exhibits non-specific hydrophobic protein-surfactant interactions, specific binding has been found in certain cases [18].

A decrease of the CMC values was found when the temperature is increased from 25°C (8.5 mM) to 45°C (6.2 mM) (data not shown). Temperature can affect cooperative hydrophobic surfactant-surfactant interactions reducing the CMC and increasing the aggregation number. These changes, normally, are related with an increase in the solubilizing capacity of the surfactant [25]. The effect of temperature and salt concentration on the solubilization process will be commented below.

Fig. 2a shows a SDS-PAGE of insoluble and soluble OTG fractions after treatment of EBs of *C. trachomatis* with two different amounts of OTG detergent. In any case, MOMP is extracted with the only action of OTG and the protein remains in the insoluble fractions. As previously commented, this protein is a component of the OM skeleton with a strong protein-protein interaction by means of disulfide bridges and, in consequence, this protein cannot be solubilized with a "mild" surfactant without the co-action of a reducing agent. The temperature variation, from 25°C to 45°C, and the increase in the electrolyte concentration, up to [NaCl] = 250 mM, did not change this result. However, the action of OTG on lipid bilayer and other membrane proteins is visible and dependent on detergent concentration, and the

presence of other proteins in the soluble OTG fraction is evident above the CMC (9 mM–0.3%). Thus, this surfactant can solubilize several peripherial proteins not covalently bound to the membrane skeleton. The extraction of other biomolecules in the soluble fraction (i.e., LPS) is also possible. These species may be found in the OM, closely associated but not covalently bound to antigenic proteins as MOMP [7]. The amphipatic nature of LPS would favor its inclusion in mixed LPS-surfactant aggregates. This aspect is relevant from an immunological point of view.

The following experiments will show the action of OTG in the presence of DTT that allows the surfactant action, breaking the cross-linking structure by means of a reduction of the disulfide bonds. As previously indicated, DTT slightly reduces the CMC of OTG (8.3 mM) and the presence of this reducing agent in the solubilization process only affects disulfide bonds of the membrane protein skeleton with no direct effect on the solubilization ability of this surfactant. A SDS–PAGE of the soluble and insoluble extracts from the solubilization experiments with different

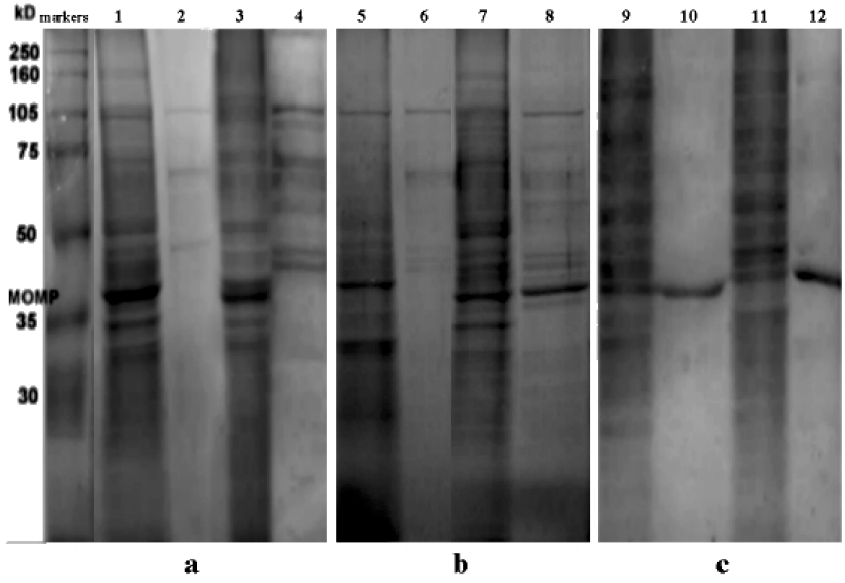

Fig. 2. SDS-PAGE of proteins of EBs of *C. trachomatis* extracted by different procedures. The odd lanes correspond to insoluble fractions, while the even lanes are soluble fractions. (a) Lanes 1-4 : Treatment in one step : lanes 1-2: 0.3 % OTG; lanes 3-4 : 1 % OTG. (b) Lanes 5-8 : Treatment in one step : lanes 5-6 : 0.3 % OTG + 10 mM DTT; lanes 7-8: 1 % OTG + 10 mM DTT. (c) Lanes 9-12 : Treatment in two steps. The insoluble fraction of lane 3, at the second step is treated with : lanes 9-10 : 0.3 % OTG + 10 mM DTT; lanes 11-12 : 1 % OTG + 10 mM DTT. The position of standard molecular weights and MOMP are indicated at the marker lane.

amounts of OTG in the presence of DTT (10 mM) is shown in Fig. 2b. In agreement with the work of Bavoil et al. [10], MOMP was partially extracted to the soluble fraction in a surfactant concentration above the CMC. However, for a concentration below the CMC, there is not enough amount of surfactant to produce protein-surfactant mixed micelles and surfactant molecules are only inserted in the membrane without extraction of biomolecules, in spite of the reduction of disulfide bonds of the special framework of the chlamydial OM brought about by DTT action.

With this treatment, MOMP, other membrane proteins and, possibly LPS, have been extracted to the soluble fraction. The extraction of LPS is not visualized with the Coomassie stain of Fig. 2a and 2b, and the solubilization of this antigenic biomolecule in the soluble OTG and OTG-DTT fractions can be proved by means of specific stains.

The presence of LPS molecules in the OTG soluble fraction was confirmed using a specific method of bacterial LPS detection in polyacrylamide gels by means of an oxidation of the LPS sample before a specific silver stain [23]. The SDS-PAGE stained with silver was later stained with blue-coomassie for detecting all protein molecules. The result of this experiment (see Fig. 3) indicates a specific smear in the position of Chlamydial LPS at the bottom of the track corresponding to the OTG soluble fraction.

In order to separate MOMP from LPS, a two step procedure was used. In the first step, treating EBs with OTG, LPS molecules and some proteins are solubilized in the OTG soluble fraction while MOMP remains in the OTG insoluble fraction (Fig. 2a). Next, by using the 1% OTG insoluble previous fraction, we can extract MOMP to the OTG-DTT soluble fraction. The results of SDS-PAGE of the second step are shown in Fig. 2c. As can be seen, a clear band around 40 kD was found in the two OTG-DTT soluble fractions, lanes 10 and 12. This band corresponds to MOMP and is similar for both concentration of OTG: 0.3% and 1%. The result for the lowest amount of surfactant, near the CMC, was at first unexpected. However, as a consequence of the action of the surfactant in the first step, the lipid bilayer gets disorganised and several biomolecules are solubilized leaving OM skeleton which is composed of MOMP to a large extent [3]. When the reducing agent broke the disulfide bonds, a lower amount of surfactant was necessary to solubilize it into the mixed protein-surfactant micelles.

The SDS-PAGE doubly stained of Fig. 3 shows the separation between chlamydial LPS, presented in the OTG soluble fraction, and MOMP, extracted in the OTG-DTT soluble fraction.

All the experiments carried out with the two step procedure showed monomeric MOMP molecules of around 40 kD isolated in the OTG-DTT soluble fraction.

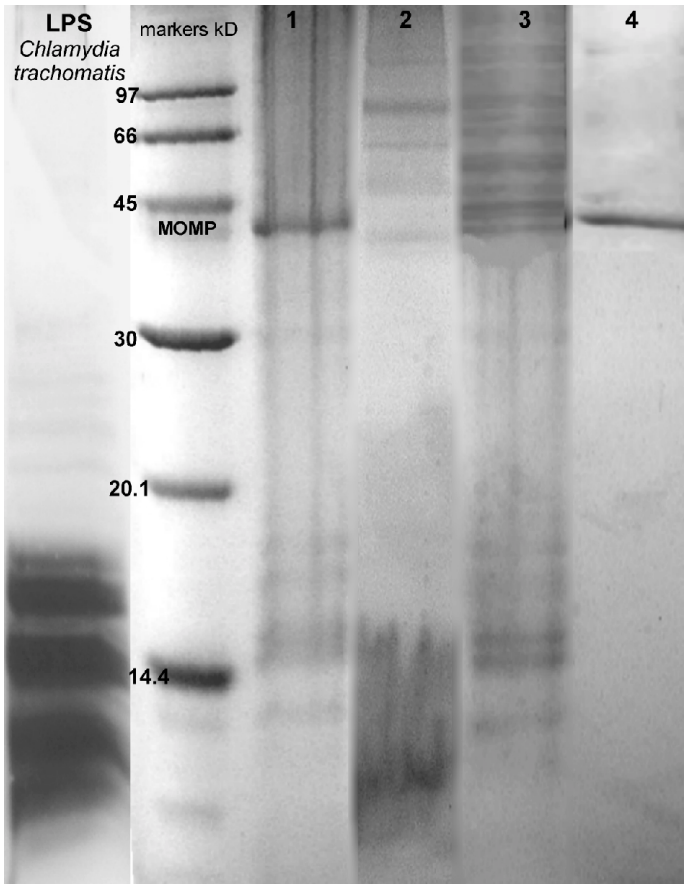

Fig. 3. SDS-PAGE with a double stain (silver and blue-coomassie). First step : lane 1, OTG insoluble fraction; lane 2, OTG soluble fraction. Second step : lane 3, OTG-DTT insoluble fraction; lane 4, OTG-DTT soluble fraction. Molecular weights of proteins and chlamydial LPS are indicated in the figure.

According to the "salting out" effect, an increase in the electrolyte concentration influences the solubilization of proteins by surfactants and the higher amount of extracted MOMP was found for the highest amount of electrolyte used: 250 mM sodium chloride. The densitometric scanning of coomasie blue-stained gels and the analysis of the protein concentration of different fractions by spectrophotometry (absorbance and BCA procedures) revealed an adequate yield in comparison with other authors [3, 10]. A summarized report of the yield and the purity of the MOMP sample obtained from the simplified two step process is shown in Table 1. These

TABLE 1.

A summary of solubilization of MOMP.

Step		Total protein[B] (mg)	Total yield[B] (%)	MOMP Purity[C] (%)
EBs (100 : 1)		548	100	25
First step	Insoluble fraction	428	–	35
	Soluble fraction	114	21	0
Second step[A]	Insoluble fraction	264	–	< 10
	Soluble fraction	120	22	> 90

[A] From insoluble fraction of the first step. [B] Total protein content was determined using BCA and spectrophotometric procedures. [C] From densitometric scanning of stained gels.

results indicate that around 90 % of MOMP was extracted from EBs using this procedure (Fig. 1c and 4).

To confirm the identity and purity, the 40 kDa band from SDS-PAGE experiments corresponding to the OTG-DTT soluble fraction, was selected for an in-gel trypsin digestion and mass spectrometry (MALDI-toff). The data searched against protein databases returned the highest and significant scanning identifications for MOMP of Chlamydia trachomatis (data not shown).

When the OTG-DTT soluble fraction comes into contact with a solid-liquid interface, competitive adsorption behavior between the two, micellar and non-micellar, species present in the fraction is expected [18]. In this situation, it is necessary to favour the interaction of MOMP with the solid-liquid interfaces of polymeric systems in order to develop immunological reactives. The non-micellar surfactant and DTT molecules from the soluble OTG-DTT fraction can be reduced by means of dialysis experiments. Due to the high CMC value of OTG, this surfactant can be easily dialyzed [17]. However, the elimination of detergent from the soluble fraction could produce instability by the aggregation of protein molecules. Thus, in previous reports [10], the removal of DTT and surfactant from the soluble fraction leads to the formation of large aggregates of chlamydial MOMP previously solubilized by a three-step procedure using two different surfactants, sarkosyl and OTG, in the presence of DTT. This phenomenon was only partially reversible even boiling under denaturant conditions. In our system, an OTG-DTT soluble fraction, obtained from the simplified two step solubilization procedure and developed in optimal experimental conditions (temperature: 37°C; 1% OTG; 10 mM DTT; and 250 mM NaCl), was dialyzed to

prove the aggregation state of the protein molecules. When the dialysis was done in deionised water, all the dialyzable components of the soluble fraction were eluted, electrolyte, DTT and surfactant, promoting the aggregation of the proteic system. SDS-PAGE experiments, after dialysis, show multi-aggregates in the OTG-DTT soluble fraction which do not enter the gel.

As previously commented, the electrolyte could play an important role in the stabilization of the MOMP-surfactant complexes, favouring a closer interaction between protein and surfactant. Thus, we have changed the dialysis medium using the same solution conditions of the second step of the solubilization procedure (saline phosphate buffer at pH 7.5 with 250 mM NaCl as the electrolyte), but without OTG and DTT. Fig. 4 shows a SDS-PAGE before and after the dialysis of the OTG-DTT soluble fraction in which MOMP is isolated. Lanes 1 to 4 show the insoluble and soluble fractions of the two phases of the isolation procedure. MOMP appears in

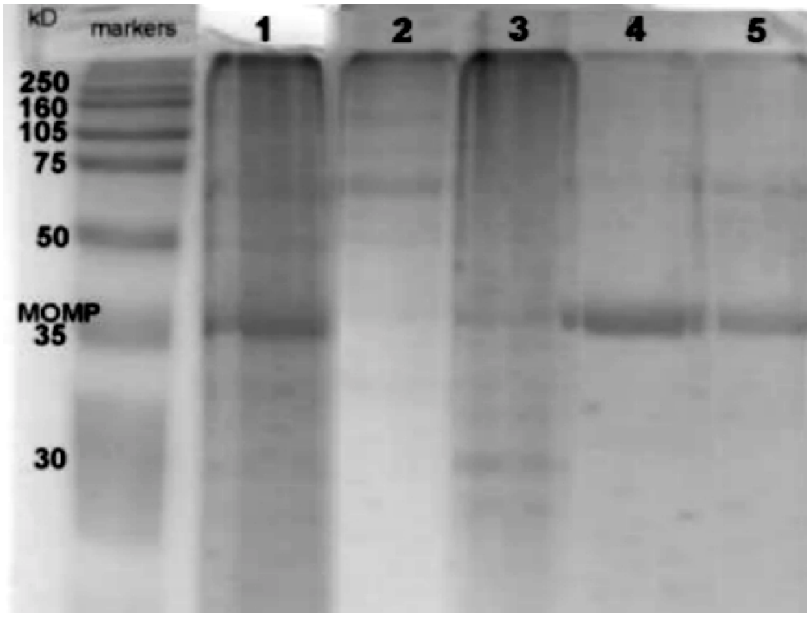

Fig. 4. SDS-PAGE of proteins of EBs of *C. trachomatis* extracted by the two step procedure. First step in lanes 1-2, lane 1 : insoluble OTG fraction; lane 2 : soluble OTG fraction. Second step in lanes 3-5, lane 3 : insoluble OTG-DTT fraction; lane 4 : soluble OTG-DTT fraction; lane 5 : soluble OTG-DTT fraction after dialysis. The position of standard molecular weights and MOMP are indicated at the marker lane. [OTG] = 1%; [DTT] = 10 mM; [NaCl] = 250 mM.

monomeric form (about 40 kD) in the OTG-DTT soluble fraction with a high degree of purity and solubilization yield. When the soluble OTG-DTT fraction is dialyzed in the conditions previously indicated, a band of monomeric MOMP was found (lane 5), although the amount of monomer in the solution is slightly reduced and a weak band appears in a position that could correspond to MOMP dimers. This result can suggest a strong interaction between protein molecules and surfactant, which could mean a partial elimination of the surfactant molecules (non-micellar detergent) after dialysis, without disrupting a larger part of the protein-surfactant complexes, and keeping the whole MOMP in a monomeric form.

Considering these results, the presence of stable and monomeric MOMP in the soluble fractions is clear. Non-ionic surfactants normally exhibit cooperative hydrophobic protein-surfactant interactions. Thus, OTG would bind to hydrophobic regions on the native protein surface to form individual protein-surfactant complexes, which could be stabilized by specific bonds. The negative protein charge at neutral pH (MOMP has an acidic isoelectric point, pI = 5) could help prevent the aggregation between these complexes.

The protocol used to purify MOMP for structural or reconstitution experiments [10] could not be employed during the isolation of MOMP for immunological applications, a situation which becomes essential for the stability of protein molecules in the solution. Next, in the final part of this work, a preliminary immunological study is developed in order to prove the immunological behaviour of the bacterial protein isolated under several experimental conditions.

In this way, the preliminary study was made throughout an immunoenzymatic assay (ELISA). The biomolecules were immobilized on the wells of microplates, as previously indicated. We have used the soluble extracts, OTG and OTG-DTT soluble fractions, of the simplified two-step solubilization method used in this work for coating microwell surfaces. The best immunological behaviour for the soluble OTG-DTT fractions was obtained with 0.3 μg of MOMP in each micro-well. We use four different plates : (1) sensitized with LPS present in the OTG soluble fraction; (2) sensitized with MOMP present in the OTG-DTT soluble fraction before dialysis; (3) the same sample after dialysis; and (4) a commercial kit from Vircell S.L. which uses MOMP molecules extracted with a method employing an anionic surfactant: sarkosyl. Several aspects of the immunological response of these different systems were studied by using different serum samples previously tested with the serological reference technique, IFA. Thus, the sensitivity of the immunological response was studied by means of positive and negative sera for *C. trachomatis*, comparing the absorbance(A) obtained in the assay for the different plates, whereas the specificity and possible cross-reactivity was analysed by using negative serum samples for trachomatis species but positive for other genus species, *Chlamydia pneumoniae*.

Fig. 5a shows the absorbance(A) obtained in the assays for the different plates and positive serum samples for *C. trachomatis*. The highest sensitivity corresponds to the OTG soluble fraction, in which, as was previously shown by SDS-PAGE and

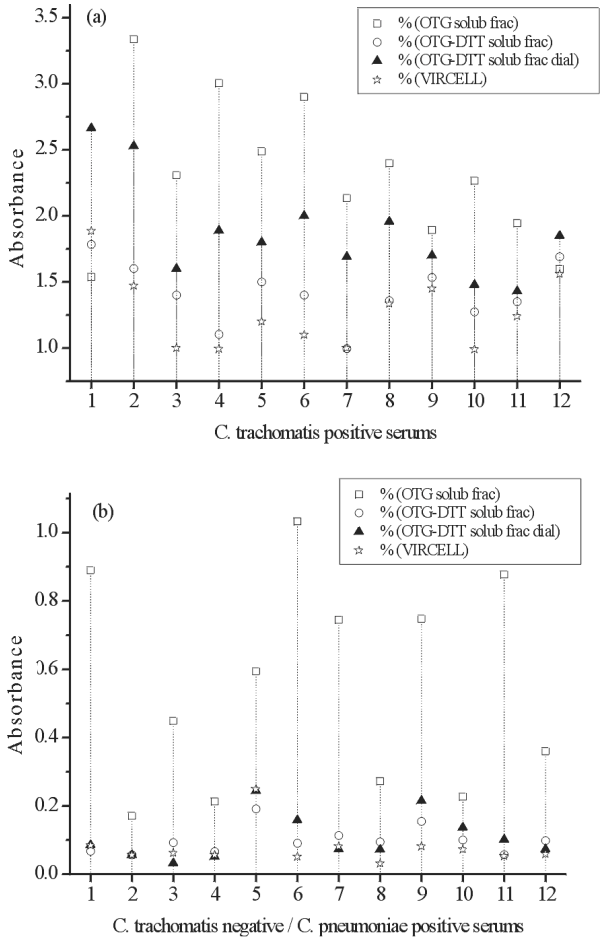

Fig. 5. Absorbance(A) measured at 450 nm of wavelength using ELISA technique. The microwells have been sensitized with the different antigens obtained in the solubilization process previously described. (□) 1 % OTG soluble fraction (first step); (○) 1 % OTG + 10 mM DTT soluble fraction (second step); (▲) 1 % OTG + 10 mM DTT soluble fraction (second step) after dialysis; (*) ELISA VIRCELL commercial test.

a) Evaluation of positive serums for *C. trachomatis*. b) Evaluation of negative serums for *C. trachomatis*, but positive for *C. pneumoniae*.

westerblotting experiments, chlamydial LPS is present. With this result the high antigenicity of this biomolecule becomes noteworthy [6]. Other remarkable result is the higher sensitivity of the OTG-DTT soluble fraction after dialysis, with respect to the same fraction before dialysis (an increase of 35 %). We have observed some MOMP aggregation after surfactant was removed by dialysis. It is possible that only the non-micellar surfactant leaves the dialysis bag without disrupting the protein-surfactant complexes in which the high hydrophobicity of the MOMP molecules allows a close interaction between protein and surfactant. The decrease of the non-micellar surfactant minimizes the competition for the solid-liquid interface during the adsorption process on the microplates and a better deposition of the MOMP molecules on the microwells. The importance of partial removing of surfactant molecules from the solution is clear from this result. Furthermore, the high immunoreactivity of MOMP in this situation would be incompatible with the formation of multi-aggregates of this protein after dialysis. The increase of sensitivity with respect to the commercially available sample is even higher (an increase of 50 %), using the same amount of soluble fractions obtained from the same amount of bacterial cells in both cases to coat the microwells.

The absorbance(A) of the ELISA assay of the different plates of serum samples negative for *C. trachomatis* is shown in Fig. 5b. As can be seen, the results for the plates sensitized with the OTG-DTT soluble fraction, with and without dialysis, and the commercial plate show a similar behavior with absorbance(A) values indicating the absence of specific antibodies of *C. trachomatis*. This result is representative of the immunological specificity of these solutions in which MOMP was isolated. However, the results for the plate sensitized with OTG soluble fraction, containing chlamydial LPS, show a given non-specificity with an increase in the absorbance(A) values for negative serum samples, and even, cross-reactivity with positive serum samples for *C. pneumoniae*. This is a consequence of the antigenic nature of chlamydial LPS, a genus antigen. This result shows the importance of the first step of the purification process, in which LPS and MOMP become separated for an optimal detection of *C. trachomatis*. Although a more exhaustive immunological evaluation is necessary, and revealing preliminary results have been obtained.

CONCLUSIONS

The two-step solubilization process in the specific conditions allows the isolation of MOMP, simplifying precedent protocols and separating this membrane protein from chlamydial LPS, in favorable structural and biological conditions for its use in immunological applications. The interaction between protein molecules and surfactant (OTG) seems to be important for the stability of the isolated protein solution. When this extracted MOMP was used in serological determinations, an adequate combination

of sensitivity and specificity was obtained. Under these conditions, this isolated protein could be used for covering the surface of colloidal particles, latex particles or colloidal gold, in order to develop rapid diagnostic tests. A more extensive immunological evaluation, in addition to a specific study on the interaction between proteins (MOMP and other typical protein molecules) and OTG, will be the object of future research.

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