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Purification of hydrophobic integral membrane proteins from *Mycoplasma hyopneumoniae* by reversed-phase high-performance liquid chromatography

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Abstract

A general and practical approach for isolating, fractionating and purifying large quantities of outer membrane hydrophobic proteins is described as applied to membrane proteins of *Mycoplasma hyopneumoniae*. Outer membrane proteins were extracted with Triton X-114 detergent and were precipitated from the detergent phase with 90% ethanol. Precipitated proteins were dissolved in 65% formic acid and separated by RP-HPLC using a formic acid–acetonitrile gradient. A M_r 48 000 protein was obtained in high yield and at greater than 90% purity by optimisation of parameters for RP-HPLC. The combination of Triton X-114 extraction followed by high resolution RP-HPLC is a novel and rapid procedure for the isolation and purification of hydrophobic proteins. Proteins purified by this approach were suitable for subsequent characterisation by direct sequencing of the amino terminus as well as generation of peptides by digestion with cyanogen bromide.

Keywords: *Mycoplasma hyopneumoniae*; Proteins; Membrane proteins; Triton X-114; Formic acids

1. Introduction

Proteins located in the outer membrane of a wide range of pathogens are of increasing interest as target molecules for the development of vaccines and diagnostic tests. Many membrane proteins are highly hydrophobic and are poorly soluble in water [1], requiring either high concentrations of solubilising agents such as urea or detergents to remain in solution. Triton X-114 (TX114) is a non-ionic detergent that solubilises proteins into protein–detergent micelles at temperatures below the cloud point of 22°C. At temperatures above the cloud point, micelles merge resulting in the partitioning of the detergent and aqueous phases which contain the hydrophobic and the hydrophillic proteins respectively [1].

Surface proteins isolated from a number of pathogenic mycoplasmas have been implicated in protection against disease [2]. Swine enzootic pneumonia, caused by *Mycoplasma hyopneumoniae*, is the most important respiratory disease of pigs world wide, resulting in significant economic losses to the pig industry [3]. The current vaccines reduce the severity of the pneumonia, but do not effectively control the disease and the fastidious growth requirements for culture of *M. hyopneumoniae* make production of these vaccines technically demanding and expensive.

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The role of the membrane proteins of *M. hyopneumoniae* in protection remains to be defined. There is therefore a need to identify, characterise and purify mycoplasmal membrane antigens for protection studies.

Selective partitioning in TX114 detergent has been used to extract integral membrane proteins from *M. hyopneumoniae* [4], and *M. hyorhinus* [5], however further methods of purification and manipulation were not described. Others [6] have fractionated proteins of *M. hyopneumoniae* by elution following separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In all these methods, the high concentrations of salts or detergents used to solubilise protein samples can interfere with subsequent characterisation of the molecules and restrict the options for further purification. There are no well-described methods for separating and purifying hydrophobic membrane proteins of mycoplasma from TX114 detergent extracts.

Hydrophobic structural polypeptides of poliovirus have been successfully separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using 60% formic acid as the mobile phase [7]. The purified polypeptides were shown to induce monospecific antibodies that could neutralise poliovirus in vitro [8]. These previous studies showed formic acid to be an excellent volatile solvent for hydrophobic proteins that can be removed easily without loss of material. We describe here an approach using RP-HPLC for the isolation and purification of hydrophobic proteins particularly those extracted with TX114, and have applied this to the purification of a M_r 48 000 membrane protein from *M. hyopneumoniae*.

2. Experimental

2.1. Mycoplasma culture

M. hyopneumoniae (Beaufort strain) was grown in culture media as described by Etheridge et al. [9]. Low-passage seed cultures (1 ml) stored at −70°C were thawed quickly and passaged into 5 ml of fresh culture medium and incubated at 37°C. These starter cultures were twice passaged before harvesting and then stored at −70°C until used.

2.2. Extraction of integral membrane proteins

TX114 (Fluka, Buchs, Switzerland) was precondensed by dissolving 1 ml of TX114 in 100 ml of cold phosphate-buffered saline (PBS) at 4°C. The solution was incubated at 37°C for approximately 6 h or until the aqueous and detergent phases separated. The upper aqueous phase was removed and the detergent phase was retained and the condensation procedure repeated three times to remove remaining hydrophillic contaminants.

Membrane proteins were extracted from *M. hyopneumoniae* using TX114 phase separation as described by Wise and Kim [4]. Briefly, 50 ml of mycoplasmal culture was centrifuged at 13 000 *g*, the cells were washed 4 times in 0.25 *M* NaCl and then resuspended in PBS to approximately 2 mg/ml of protein estimated by the BCA protein assay kit (Pierce, Rochford, IL, USA). Precondensed TX114 was added to the mycoplasmal suspension to give a final detergent volume of 1% (v/v), incubated at 4°C overnight with gentle mixing and then centrifuged at 13 000 *g* for 5 min at 4°C to remove insoluble material. The resulting supernatant was incubated at 37°C for 15 min to induce the separation of the detergent phase containing hydrophobic membrane proteins and the aqueous phase. The upper aqueous phase was removed and the detergent phase reconstituted to the original volume with PBS. Extraction of the TX114 phase was repeated three times. Proteins were precipitated from the detergent phase by addition of ethanol to 90% (v/v) with the addition of 1 μg/ml of dextran (M_r 79 000, Sigma Aldrich, Australia) as carrier. The precipitate was collected by centrifugation at 13 000 *g* for 40 min after incubation overnight at −20°C. The pellet was washed once in 90% ethanol and stored at −20°C until required.

2.3. RP-HPLC

A reversed-phase column packed with Vydac C_4 (250×1.6 mm), 5 μm particles with 30 nm pores (MZ-Analytical, Mainz, Germany) was attached to a Beckman HPLC system comprising of two Model

114 M solvent-delivery pumps, a Model 340 organiser with injection valve and gradient mixer chamber, and a Model 165 variable-wavelength detector. The RP-HPLC column was kept at room temperature during column runs. Chromatographic profiles were analysed with the software package, Varian Star Integrator (Varian Instruments, Melbourne, Australia).

The proteins extracted in the TX114 phase and precipitated by 90% ethanol were redissolved in 65% (v/v) formic acid (Rectapur, Prolabo, Paris, France). The undissolved material was removed by centrifugation at 13 000 *g* for 15 min. A total of 800–900 μg protein was injected onto the column. The mobile phases consisted of 65% formic acid–35% Milli-Q water (buffer A) and 65% formic acid–35% acetonitrile (buffer B). The column was injected with 20% buffer B at a flow-rate of 0.4 ml/min. Gradients were run from 20 to 100% buffer B, eluted peaks were detected at 278 nm and 1 ml fractions were collected. Formic acid was removed under vacuum using the DC 40 centrifugal vacuum concentrator (Dynavac Engineering, Melbourne, Australia).

2.4. SDS–PAGE

SDS–PAGE was carried out according to the method of Laemmli [10] using a mini Protean II gel apparatus (Bio-Rad, Sydney, Australia). All samples were run under reducing conditions and gels were stained with either Coomassie Brilliant Blue R250 (Bio-Rad) and/or silver stained as described by Heukeshoven and Dernick [11]. Fractions corresponding to UV absorbing peaks were pooled, dried under vacuum, reconstituted in reduced sample buffer [2% (w/v) SDS–10% (w/v) glycerol–62.5 m*M* Tris–HCl–50 m*M* 2-mercaptoethanol] and run on SDS–PAGE. Proteins were then electro-blotted onto polyvinylidene difluoride membrane (PVDF) (Immobolin P, Millipore, Melbourne, Australia) in CAPS buffer (10 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid–10% (v/v) methanol pH 11.0) at 100 V for 30–45 min [12] using a mini Trans-blot electrophoretic transfer apparatus (Bio-Rad). The transferred proteins were stained lightly with Coomassie blue and cut out of the membrane for amino acid sequencing. An automated gas-phase sequencer Model 476A (Applied Biosystems, Foster City, CA, USA) was used to determine the amino acid sequence of the amino (N) terminus of transferred proteins.

2.5. Two-dimensional gel electrophoresis

Mycoplasma membrane proteins (2.5 μg) extracted by TX114 and isoelectric focusing standards (Bio-Rad) were electrophoretically separated according to the manufacturers instructions on Immobiline DryStrips with a pH gradient of 3 to 10 (Pharmacia LKB Biotechnology, North Ryde, Australia). The second dimension was carried out using 12.5% SDS–PAGE gels on a Protein II xi cell apparatus (Bio-Rad) and gels were silver stained.

2.6. Cyanogen bromide (CNBr) digestion

Digestion of purified proteins with CNBr was used to generate peptides from which further sequence data could be obtained [13]. Protein purified by RP-HPLC was reconstituted in 70% formic acid and a small crystal of CNBr was dissolved in the protein solution. Digestion was carried out in the dark at room temperature for 12 h and stopped by evaporating off the CNBr and formic acid. A modified SDS–PAGE system was used to separate peptide products for sequencing [14]. Gels were prepared in three layers, a stacking layer of 4% (w/v) acrylamide, a "spacer" layer of 10% acrylamide and a separating layer of 16% acrylamide–8% (w/v) glycerol. The "spacer" and separating layers were buffered with 0.4 *M* Tris pH 8.8–0.1% (w/v) SDS and the stacking layer was buffered with 0.15 *M* Tris pH 6.8–0.1% (w/v) SDS. Peptides were electrophoretically separated using running buffer (19 m*M* glycine–2.5 m*M* Tris–0.01% (w/v) SDS) at 100 mA, transferred onto PVDF membrane and stained with Coomassie. Relevant bands were cut out for sequencing as described above.

2.7. Densitometry

SDS–PAGE gels that had been stained with Coomassie blue were read by computerised imaging using Photoshop version 2.5 (Adobe Systems, Mountain View, CA, USA) and the intensity of protein was analysed by the computer package NIH Image

version 1.55 [15]. Protein concentrations were estimated by comparing with a standard of bovine serum albumin which was included in the same gel.

3. Results

3.1. Isolation of proteins solubilised by TX114

Membrane proteins extracted from *M. hyopneumoniae* using TX114 detergent were found to be poorly soluble in water. Further purification by conventional ion-exchange or size-exclusion chromatography in the presence of non-ionic detergents such as Triton X-100 or salts such as urea resulted in problems of poor solubility and very significant loses of 90% or more (data not shown). RP-HPLC with a formic acid solvent system was developed and applied to overcome these difficulties.

Proteins obtained in the TX114 detergent phase and proteins remaining in the aqueous phase were precipitated from solution by 90% ethanol and analysed by SDS–PAGE. Four major protein bands with approximate molecular sizes of between ca. M_r 74 000 and 48 000 were identified in the detergent phase and were selected for further purification by RP-HPLC; these proteins were not present in the aqueous phase (Fig. 1). The N-terminal sequence of the M_r 48 000 protein fractionated by TX114 extraction was determined directly on electro-blotted protein. Nine amino acids were identified: AGNGQTEXXST; residues 8 and 9 could not be assigned (denoted X).

3.2. Protein purification by RP-HPLC

Membrane proteins of *M. hyopneumoniae* were separated by RP-HPLC using formic acid–water (65:35; buffer A) and formic acid–acetonitrile (65:35; buffer B) as the mobile phases. Samples were injected with 20% buffer B, which was increased to 35% between 5 to 10 min after sample injection, and then increased to 75% over the next 60 min. The column was washed for 5 min with 100% buffer B before re-equilibrating with 20% buffer B (see Fig. 2A). Aliquots of the collected fractions were dried down under vacuum and analysed on SDS–PAGE. The M_r 74 000 resolved into upper and

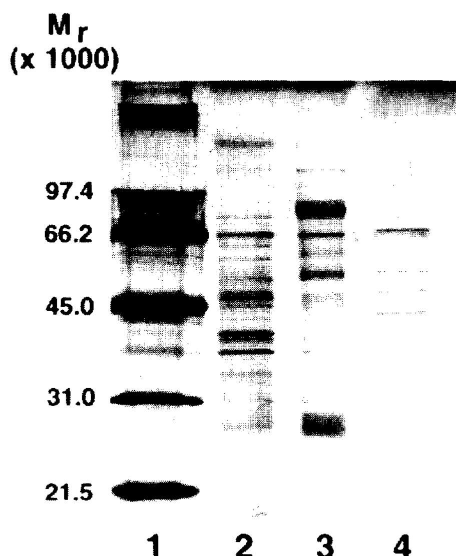

Fig. 1. SDS–PAGE (12.5% acrylamide) of TX114 phase separated protein from *M. hyopneumoniae* double stained with Coomassie blue followed by silver staining. Lane 1, molecular mass standards (Bio-Rad). Lane 2, whole mycoplasma solubilised in SDS. Lane 3, proteins precipitated from the aqueous phase of TX114 extraction. Lane 4, proteins precipitated from the detergent phase of TX114 extraction.

lower bands which were separated at 51 min and 74 min respectively, the M_r 52 000 protein eluted at 54 min and the M_r 48 000 protein eluted at 48 min after injection of the sample (Fig. 3). Peak fractions of the M_r 48 000 protein were dried down and pooled. The second stage of the RP-HPLC purification of the M_r 48 000 was run over 80 min with modification of the gradient. Five min after the sample was injected, buffer B was increased from 20 to 55% over 10 min and from 55 to 70% over 55 min (Fig. 2B). Densitometry measurements showed that the M_r 48 000 protein was 50% to 70% pure in the peak fraction (47.5 to 50 min) collected from the first RP-HPLC step, and approximately 40% of the total M_r 48 000 protein was eluted in this single fraction (see Fig. 3). A second column run with the M_r 48 000 protein fraction after pooling and concentrating the sample from the first chromatographic step resulted in greater than 90% purity (Fig. 4).

The M_r 48 000 protein obtained from the second chromatography run was digested with cyanogen

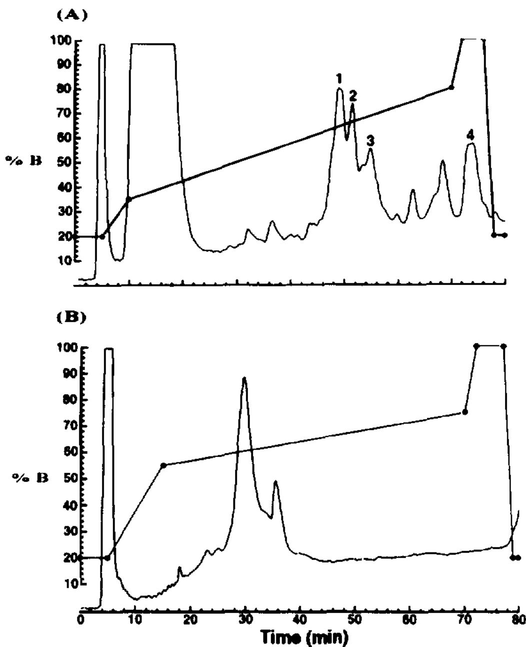

Fig. 2. (A) Chromatogram showing the RP-HPLC purification of proteins from *M. hyopneumoniae* isolated by TX114 phase separation. Elution peaks of the M_r 48 000, 74 000 (upper band), 52 000 and 74 000 (lower band) proteins are indicated by numbers 1, 2, 3 and 4, respectively. The column was injected with 800 to 900 μg of protein and the wave length of the detector was 278 nm. (B) Chromatogram of the second RP-HPLC purification of the M_r 48 000 protein.

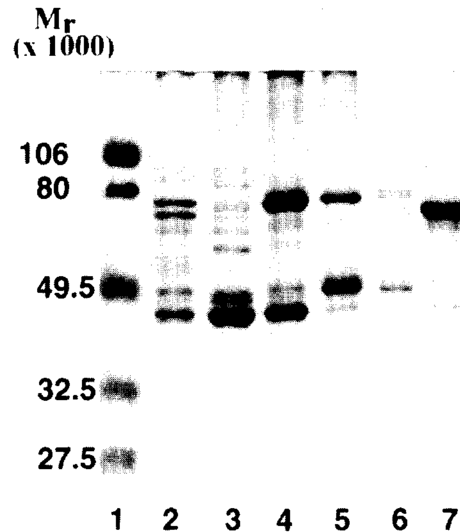

Fig. 3. SDS–PAGE (12.5%) of the eluted fractions from RP-HPLC purification. Lane 1, prestained molecular mass markers (Bio-Rad). Lane 2, preload sample of *M. hyopneumoniae* proteins solubilised from the TX114 detergent phase. Lane 3, fraction collected at 47.5 to 50 min. Lane 4, fraction collected at 50 to 52.5 min. Lane 5, fraction collected at 52.5 to 55 min. Lane 6, fraction collected at 55 to 57.5 min. Lane 7, fraction collected at 72.5 to 75 min.

bromide. Four fragments of ca. M_r 21 000, 19 000, 13 000 and 9000 stained with Coomassie blue on a step gradient gel and these peptides were blotted onto a PVDF membrane for N-terminal amino-acid sequencing. The N-terminal sequence of the M_r 48 000 protein was located on the M_r 13 000 fragment and 25 amino acids were sequenced from this polypeptide as AGNGQTESGSTNDEKPQAEIP-AHKV.

3.3. Two-dimensional gel electrophoresis

Proteins extracted by TX114 detergent were further characterised by two dimensional gels. The M_r 48 000 protein was identified as a single spot with an estimated isoelectric point of ca. 6.2.

4. Discussion

Previous applications of TX114 for the solubilisation of membrane proteins have not described procedures for further manipulation of these hydrophobic macromolecules [3–5]. Membrane proteins extracted from *M. hyopneumoniae* by TX114 were conveniently obtained free of detergent by precipitation with 90% ethanol and were suitable for analysis by SDS–PAGE and electro-blotting or solubilisation with 65% formic acid and direct application onto RP-HPLC. The M_r 48 000 protein fractionated by RP-HPLC using 65% formic acid as the mobile phase was approximately 70% pure after a single chromatography step and a purity of over 90% was achieved after a second chromatography step. Although 40% of the M_r 48 000 protein was collected in a single fraction, up to 60% of this protein was eluted in neighboring fractions containing other major contaminating proteins. This probably reflects the fact that, either under the

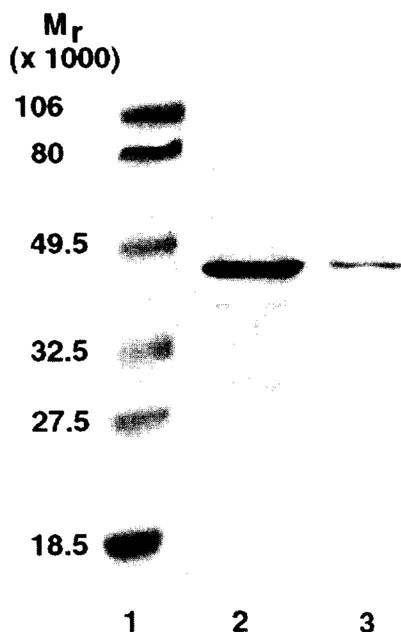

Fig. 4. SDS–PAGE (12.5%) of the purified M_r 48 000 protein. Lane 1, prestained molecular mass markers (Bio-Rad). Lane 2, the M_r 48 000 protein fractionated by a single step RP-HPLC procedure. Lane 3, the M_r 48 000 protein purified by a two-step RP-HPLC procedure.

chromatographic conditions the protein was not completely unfolded, or that secondary modifications such as the addition of lipid were affecting its chromatographic behavior. Proteins in the TX114 phase have been shown to be integral membrane proteins with highly hydrophobic domains [16]. Mycoplasmal surface proteins may interact with the cell membrane via such hydrophobic domains, or be anchored via lipid modification [17]. Previous studies [4] have described a M_r 44 000 lipoprotein extracted with TX114 from *M. hyopneumoniae*; however, the relationship of that protein to the M_r 48 000 protein described here is not known.

Yields of the TX114 extracted proteins of approximately 70% were significantly higher than yields of <10% obtained with other detergents or urea as solubilising agents. Studies with the highly hydrophobic structural proteins from poliovirus showed approximately 90% recovery [8]. Similarly, hydrophobic proteins from bacterial membranes have been purified by size exclusion chromatography using 50% formic acid–25% acetonitrile–15% water as the mobile phase with a recovery of 60–77% of the original protein mixture [18].

No degradation was observed of the M_r 48 000 protein or of the other major proteins in the TX114 extract following solubilisation and chromatography in 65% formic acid. The N-terminal amino acid sequence of the M_r 48 000 protein determined after SDS–PAGE separation and electro-blotting on a nylon membrane or after purifying by RP-HPLC in 65% formic acid followed by CNBr digestion were identical, therefore, indicating that there was no effect on the N-terminal sequence after solubilising in formic acid. Aspartic acid–proline peptide bonds are cleaved when heated in high concentrations of formic acid but previous studies with proteins from Herpes simplex virus showed no degradation after storage at 37°C for 18 h or at −20°C for two months in 50% formic acid [19]. Disulphide bonds of proteins remain intact in 80% formic acid [7], although secondary structure may be reversibly altered, depending on the hydrophobicity of the molecule and concentration of acid [20]. Searches of protein databases showed the NH_2-terminal and internal peptide sequences obtained from the purified and CNBr-digested M_r 48 000 protein to match the protein sequence predicted by the cloned gene encoding a M_r 46 000 protein from *M. hyopneumoniae* [21]. This provides further evidence that exposure of proteins to 65% formic acid does not lead to unwanted modifications or degradation. Antibodies obtained by vaccination with formic acid-RP-HPLC purified M_r 48 000 protein recognised native antigen (data not shown) and others [8,18] have raised neutralising antibodies by immunisation with proteins purified by this approach, but whether such antibodies recognised conformational or linear epitopes was not determined.

In conclusion, we have demonstrated the general utility of an approach for purifying highly hydrophobic membrane proteins by extracting with TX114, precipitating the proteins with 90% ethanol, re-solubilising in 65% formic acid and subsequent purifying by RP-HPLC with high yield of product. This method can be applied to hydrophobic proteins or peptides solubilised directly in formic acid, or for manipulation of membrane proteins following extraction with ionic or non-ionic detergents. Proteins

so purified are suitable for further characterisation, vaccination studies and for use in diagnostic tests.

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