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ISOLATION AND PURIFICATION OF MAJOR OUTER MEMBRANE PROTEINS FROM *BRUCELLA ABORTUS* S-99

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ABSTRACT

Isolation and purification of major outer membrane proteins (OMP) from the cell wall envelope of *Brucella abortus* S-99 were achieved by sonication, solubilization and membrane fractionation in the presence of non-ionic detergent (Tx-100) and lysozyme treatments, followed by ultracentrifugation.

The crude OMP was treated with trypsin to free the preparation from any other protein contaminants. The OMP preparation was purified by column chromatography on Sephacryl S-200. Three major symmetrical peaks emerged from the column with kav values of 1.81, 2.42 and 2.56 in succession in addition to a few closely related minor peaks. Characterization of crude OMP on SDS-PAGE showed 13 protein bands. The three major peaks 1,2 and 3 were subjected to SDS-PAGE separately and the molecular weights of peaks 2 and 3 were calculated to be 26 and 38 kDa, respectively and the first peak was further resolved into two subfractions with molecular weights of 62 and 67 kDa.

However, after treatment of OMP with trypsin the number of bands were reduced to one prominent band with a molecular weight of 38 kDa and a thinner band of 41 kDa.

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INTRODUCTION

Brucella abortus is an intracellular bacterium which resides in the macrophages of humans and animals where they are inaccessible to antibodies and complement components.[2,13] Despite a great deal of research regarding the molecular composition of this microorganism,[3,4,6,8,11] there are still areas of major importance where our knowledge is far from complete. As a result, no effective vaccine is as yet available for prevention of this infectious bacterial disease and the role of protein components of the bacterium in the establishment of immunity and pathogenesis remains unclear.

Since this bacterium lacks a capsule, flagella and pili,[2,13] the outer membrane proteins are the essential components of the bacterium which are responsible for induction of immunity in the host. The proteins, unlike the polysaccharides, are thymus-dependent antigens which are necessary for induction of cell-mediated immunity (CMI) against intracellular pathogens.[11,21] The cell wall of *B. abortus* is resistant to ionic and non-ionic detergents, ethylene diamine tetra-acetic acid (EDTA), and trypsin[12,14,22] and is more hydrophilic than that of *E. coli* and its proteins are similar to the matrix proteins of other Gram–negative bacteria.[6,15,17,18]

At present there are different views regarding the molecular characteristics of the OMP of *B. abortus* and the methods of its purification. Differential extraction procedures have been used to characterize these proteins.[6,9-11,15,22,23]

Using sodium dodecyl sulfate (SDS), at a temperature of up to 100°C, and lysozyme, Dubray and Charriaut (1983) have succeeded to obtain three protein components with molecular weights of 15, 35 and 37 kDa,[5,6,23] whereas the proteins which Verstrate and coworkers (1982) isolated using lysozyme and ionic detergent consisted of fractions with molecular weights of 30, 43 and 94 kDa.

Verstrate and Winter (1984) were also able to isolate proteins with molecular weights of 38 and 41 kDa whereas Maria and Gomez (1986) using SDS and trypsin isolated a few 35 kDa proteins and smaller protein molecules from the outer membrane of *B. abortus*.

In the present study, we have employed sonication and lysozyme to disrupt the bacterial cell wall. The outer membrane proteins were isolated by ultracentrifugation in the presence of a detergent, followed by chromatography on Sephacryl S-200 column. Four protein fractions were obtained with relative molecular weights of 26,38,62 and 67 kDa.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The standard smooth strain of *B. abortus* S-99 was used. This strain was cultured in Brucella agar (Difco) at 37°C. The bacteria were harvested at their log phase and were killed using 0.5% formalin, followed by heating in a 60°C water bath for one hour. The killed bacterial cells were washed twice with 10 mM phosphate buffered saline PBS, pH 7.2, and the pellet was kept at- 20°C until use[22] and the extracted material was purified as follows.

Tris-hydrochloride, pH 7.5, was added to the preparation (20 mL/g wet weight). The extract was treated with a solution containing 1 mg/100 mL each of DNase and RNase and incubated at 37°C for 2h. After incubation, the material was treated with 0.5 mM phenyl-methyl-sulfonyl-fluoride (PMSF).[9,22]

The suspension was then subjected to sonic oscillation for 25 minutes using B-Brown 5 T probe Tip sonicator in ice. The disrupted bacterial suspension was spun at 3000 g for 20 min at 4°C and the supernatant fluid was re-centrifuged at 150,000 g for 60 min at 4°C. The pellet which consisted of crude membrane was suspended in 10 m M Tris hydrochloride buffer, pH 7.5, and treated with 2% Triton X-100 for 10 min at 23°C to solubilize the cytoplasmic membrane.[17,19] The resulting solution was spun at 50,000 g for 45 min at 4°C and dialyzed against 10 mM Tris HCl buffer for 72 h with repeated changes of the buffer. The retentate which consisted mainly of external bacterial membrane was treated with lysozyme (1m per 50 mg protein) for 24 h at 37°C.[22]

A sample of crude OMP was treated with trypsin 1mg/ 10mg protein[9] and fractionated on a column (1.6×45cm) of Sephacryl S-200 and eluted with 10 mM Tris HCl buffer containing 0.25 M NaCl, 3 mM $NaNO_3$ and 0.1% SDS with a flow rate of 7 mL/h at room temperature. Blue dextran was used to determine the void volume and the kav for each peak was calculated.

The relative molecular weights of the isolated proteins were determined by SDS-PAGE.[12]

Pyrogen test

Pyrogen test was employed to determine the presence of minute contamination with lipopolysaccharide (LPS) in the purified cell wall protein preparation. Briefly, 10 and 25 µg of each cell wall protein sample was injected intravenously into rabbits. The body temperature of each rabbit was determined at 1, 2 and 3 hour intervals after injection.

RESULTS

Outer membrane proteins of *B. abortus* were isolated by sonication, followed by ultracentrifugation, detergent solubilization and enzyme treatments, and afterwards chromatography. A sample of crude OMP was treated with trypsin and analyzed by SDS-PAGE, revealing a single protein band with a molecular weight of 38 kDa and a thinner band of 41 kDa (Fig. 1).

Another sample of the same crude OMP which had not been digested with trypsin was lyophilized. Pyrogenicity

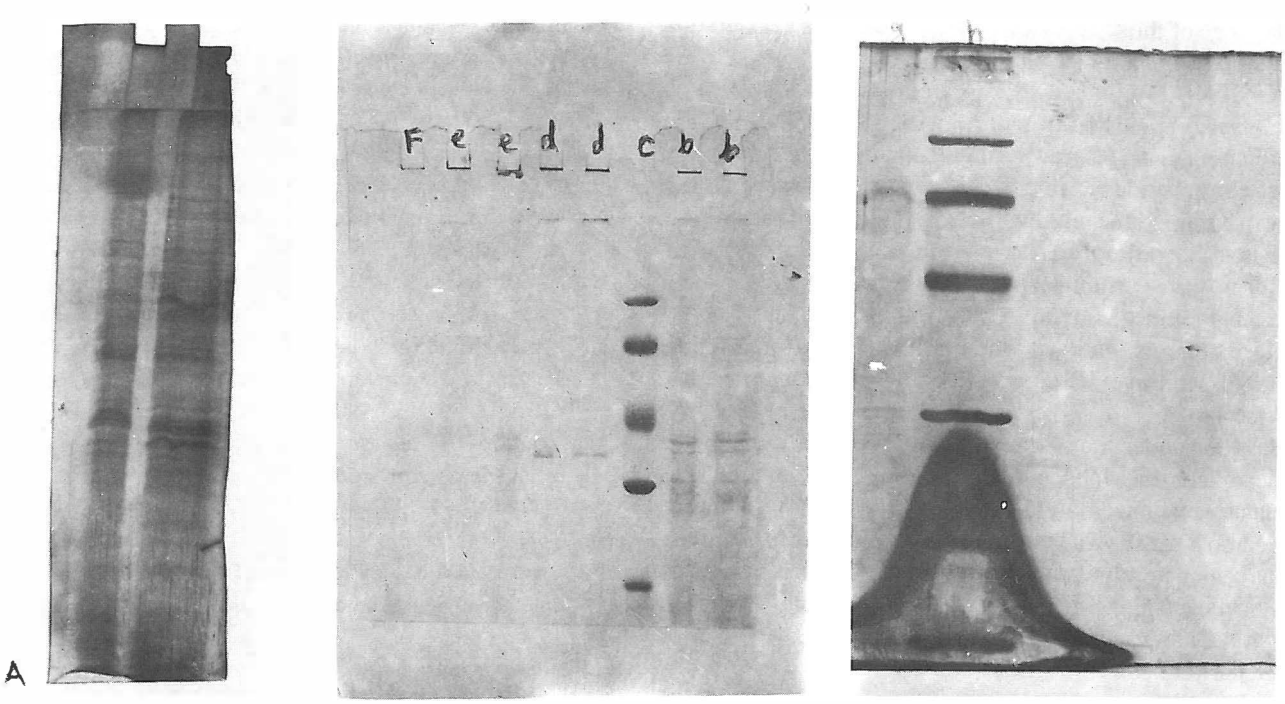

Fig. 1. SDS-PAGE profiles of *B. abortus strain* 99 outer membrane proteins. **lanes: a:** Crude membrane preparation, **b:** Semipurified OMP, **c:** Standard, **d:** Trypsin digestion products, **e:** Second peak, **f:** Third peak. **g:** First peak, **h:** Standard Coomassie brilliant blue was used for staining.

test of this sample indicated that the preparation lacked LPS. Analysis of this sample by SDS-PAGE revealed more than 10 different protein bands which visualized on the gel. The protein bands corresponded to relative molecular weights of 26-29, 31-32, 38, 41, 55, 62, 67, 72, 88 and 94 kDa, respectively.

The lyophilized OMP samples which had not been treated with trypsin were also chromatographed on Sephacryl-200 column and revealed three distinct peaks (Figs. 2, 3).

SDS-PAGE analysis of the pooled fractions from each peak revealed only four distinct bands with relative molecular weights of 26, 38, 62 and 67 kDa.

DISCUSSION

Isolation of OMPs of *B. abortus* cell wall could lead to the separation of macromolecules which may be useful in the prevention and treatment of brucellosis. The proteins, unlike the polysaccharides, are thymus-dependent antigens and thus are necessary for induction of CMI against intracellular pathogens.[1] Mareno et al. (1979) have reported that the OMP of the wall of brucellae are tightly bound to LPS. However, in the present study a combination of sonication, followed by enzyme and detergent treatment was found to be effective for isolation of the peptidoglycan of the outer membrane proteins of *B. abortus*.

The molecular weights of the proteins isolated were in

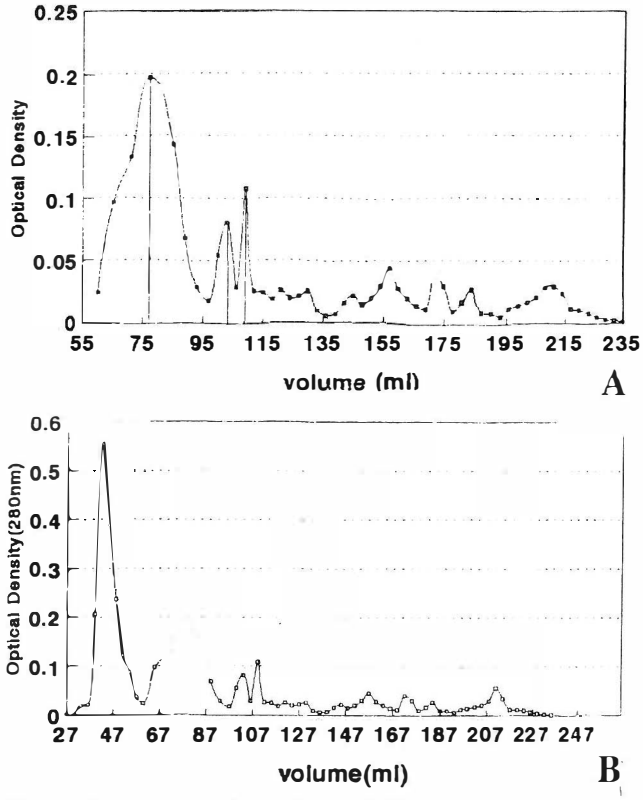

Fig. 2. Chromatographic profile of OMP of *B. abortus* S-99 on Sephacryl S-200.

A. Outer membrane proteins.

B. Dextran blue + outer membrane proteins.

the range of those reported by James Douglas et al. (1984) and Verstrate et al. (1984).

SDS-PAGE analysis revealed that the OMPs of *B. abortus* consisted of more than 13 proteins with a few prominent ones. However, fragmentation or aggregation of protein molecules during the process of isolation could not be ruled out. Since the OMPs of Gram-negative bacteria are resistant to proteolysis, trypsin treatment of the purified protein gave a number of contaminated protein bands, except for those with molecular weights of 38 and 41 kDa. This finding is similar to the observation of Gomez et al. (1986). Moreover, Douglas et al. (1984) have reported that these proteins may be porin-like molecules which are classified under the so-called group 2 proteins of the outer membrane that induce both humoral and cell-mediated immune responses (Verstrate and Winter, 1984). Dubray et al.[6] also have shown that the cell wall protein of *B. abortus* can confer immunity against brucellosis in mice. However, a dominant antigen among the cell wall antigens of *B. abortus* which can potentially induce a protective response has not yet been identified.

Brucellosis is still a health hazard in many countries and progressive research programs are necessary to pave the way for an inexpensive method of control and eradication of the disease in endemic areas. Since globular proteins are more immunogenic than fibrous proteins and OMPs are globular proteins exposed on the surface of bacteria,[7] we are at present actively involved in resolving the role of these proteins in the induction of cell-mediated immunity by attempting to identify a protein molecule or combination of protein molecules capable of inducing a protective immune response against brucellosis.

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