Preliminary Extraction and Identification of the 44.5 kDa Outer Membrane Proteins Isolated from Bovine *Fusobacterium necrophorum* (AB)

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Abstract *Fusobacterium necrophorum* (AB) in the pharynx, respiratory tract, female reproductive tract or urinary system is the causative agent of footrot and hepatic abscesses in animals and acute Lemierre's syndrome in humans. Current methods do not effectively protect animals and humans against *F. necrophorum* (AB). The outer membrane proteins (OMP) of *F. necrophorum* (AB) can be used as new material to protect against the diseases induced by *F. necrophorum* (AB). The aim of this study was to extract OMP and examine the immunogenic response of OMP. The preliminary extraction of OMP of *F. necrophorum* (AB) was identified by SDS-PAGE and stained by Coomassie Brilliant Blue R-250 (CB B R-250) and silver staining methods. The results showed that only a major band of 44.5 kDa was observed when staining the gel using CB B R-250. This band represented the target protein. In contrast, many small bands were observed by the silver staining method. The OMP also exhibited immune biological activities according to western blot analysis. The brightest band among the multi-banding observed was the OMP. Thus, the OMP was obtained and had immunogenic activity. The results provide a new direction to protect

animals and humans against *F. necrophorum* (AB) in the clinical setting.

Keywords Preliminary extraction · 44.5 kDa · *Fusobacterium necrophorum* (AB) · Outer membrane proteins

Introduction

Fusobacterium necrophorum, a gram-negative anaerobic bacillus, is a primary pathogen of local abscesses, throat infections or systemic life-threatening diseases [1]. *F. necrophorum* is classified into three biovars: A (the most virulent *necrophorum* subspecies), B (the virulent *funduliforme* subspecies) and AB (the more virulent biovar separated from sheep or deer) [2]. Madhan et al. [3] reported that outer membrane proteins (OMP) from three biovars of *F. necrophorum* had different molecular weights. OMP exists within the membrane of bacteria and plays an important role in *F. necrophorum* attachment to eukaryotic host cells, and is considered as the first step of infection [4]. OMPs are similar to fimbriae and flagellum [5, 6]. OMPs are immune proteins for *Actinobacillus pleuropneumoniae*, and expected to aid in the development of new vaccines against *A. pleuropneumoniae* [7]. Krishnan et al. [4] have reported that OmpA and OprF (a type of OMP) play a key role in bacterial infection. Wu et al. [8] reported that the OMP of the gram-negative bacteria may lay a foundation for a theory in the clinical setting. OmpA is a promising candidate for a subunit vaccine against *Haemophilus parasuis* [9]. However, there remains limited information about the immunity of OMP of *F. necrophorum* (AB). In this report, the OMP of *F. necrophorum* (AB) was preliminarily extracted and identified by SDS-PAGE.

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The immunogenic activity of the protein was examined by western blot analysis.

Materials and Methods

Bacterial Strains and Cultural Conditions

Fusobacterium necrophorum (AB) (Institute of Special Economic Animal and Plant of Science, Chinese Academy of Agricultural Science, Changchun, China) was inoculated in brain heart infusion (BHI) broth and grown at 37 °C for two days under anaerobic conditions. This culture was transferred to ten times the volume of the former BHI broth and cultivated for a further two days with daily transference in the above anaerobic environment. The bacteria were centrifuged at 6,000 rpm for 20 min at 4 °C, washed three times with 0.8 % saline and stored at –80 °C.

Preparation of OMP

Three methods were used to obtain the OMP of *F. necrophorum* (AB), and the samples were identified by SDS-PAGE. By comparing the yields, the method giving the highest yields was used to obtain the OMP of *F. necrophorum* (AB).

Comparison of the Three Methods

Protein samples were prepared according to three methods. The sediment of lane A (14,000 rpm, 4 °C, 3 h) was obtained by method two, that of lane B (15,000 rpm, 4 °C, 3 h) was obtained by method three, and that of lane C (6,000 rpm, 4 °C, 30 min) and the supernatant of lane D (6,000 rpm, 4 °C, 30 min) was obtained by method one. The methods were as follows:

Method One

Although Madhan et al. [3] proposed this method, we modified some procedures based on the present conditions in our laboratory. The lyophilized cells (200 mL culture) were suspended in 10 mL of 20 % sucrose dissolved in 10 mM TE (5 mM EDTA, 1 mM β -mercaptoethanol) supplemented with 50 μ L lysozyme (10 mg/mL) at 30 °C and this solution was incubated for 15 min. The cell suspension was treated with an ultrasonic wave at an output voltage of 320 W for 40 min (pulse on 6 s, pulse off 6 s) with the addition of 1 mM PMSF (phenylmethanesulfonyl fluoride). After the normal cells were removed at 4 °C by centrifugation (6,000 rpm) for 30 min, the supernatant (6,000 rpm, 4 °C, 30 min) was packed on ice. The sediment (6,000 rpm, 4 °C, 30 min) was suspended in 10 mL

of 2 % Triton X-100 dissolved in 10 % SDS and stored at –80 °C.

Method Two

This method was performed according to Zhu [10]. The lyophilized cells (200 mL culture) were suspended in 10 mL of 100 mM Tris–HCl (10 mM MgCl_2) supplemented with 100 μ L lysozyme (10 mg/mL) at 30 °C and incubated for 15 min. PMSF (1 mM) was then added and the cell suspension was disrupted with an ultrasonic wave at an output voltage of 520 W for 40 min (pulse on 6 s, pulse off 6 s). The sediment (14,000 rpm, 4 °C, and 3 h) was suspended in 10 mL of 100 mM Tris–HCl (10 mM MgCl_2) and stored at –80 °C.

Method Three

Some modifications in this method were made to the related reagents proposed by Srividhya et al. [11] and the centrifugation force and time suggested by Zhu [10]. under the present conditions in our lab. The lyophilized cells (200 mL culture) were suspended in 10 mL PBS (pH 7.4). The suspension was treated with an ultrasonic wave at an output voltage of 520 W for 40 min (pulse on 6 s, pulse off 6 s). Following the removal of the normal cells at 4 °C with a low-speed centrifugation step (1,000 rpm, 30 min), the remaining supernatant (1,000 rpm, 4 °C, 30 min) containing ruptured cells was centrifuged at 15,000 rpm for 30 min. The sediment (15,000 rpm, 4 °C, 30 min) obtained was suspended in 20 mL of 0.5 % sodium lauryl sarkosinate (SLS, Yi Ji Industrial Co., Ltd. Shanghai, China) aqueous solution, and the suspension was gently stirred at 25 °C for 30 min to solubilize the inner membrane proteins [12]. The suspension solution was then centrifuged at 15,000 rpm for 30 min and the sediment collected. The sediment (15,000 rpm, 4 °C, 30 min) was suspended in 20 mL of 2 % lithium dodecyl sulfate (LDS; Yi Ji Industrial Co., Ltd. Shanghai, China) dissolved in 20 mM HEPES–LiCl (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–lithium chloride) buffer (Hua Mei Biological Engineering Co., Ltd. Shanghai, China) at 4 °C and incubated for 10 min. The pH was adjusted to 7.4 with sodium hydroxide. The suspension was then centrifuged at 15,000 rpm for 3 h at the same temperature. The sediment (15,000 rpm, 4 °C, and 3 h) was suspended in 5 mL of 20 mM HEPES and stored at –80 °C.

Cooperation of Sample and Centrifugation Times

Initially the preparation of the OMP was obtained by method two according to Zhu [10]. The centrifugation speed of the sample was the same, but the centrifugation

time was 3 h and 30 min, respectively. Lane A (sediment, 14,000 rpm, 4 °C and 3 h), lane B (supernatant, 14,000 rpm, 4 °C and 3 h) and lane C (supernatant, 14,000 rpm, 4 °C and 30 min) were used to compare with lane M (Marker).

Identification of OMP by SDS-PAGE

At 4 °C the sample and centrifugation time adopted to do this experiment were the sediment at 15,000 rpm for 3 h. Madhan et al. [3] reported that OMP of *F. necrophorum biovar (AB)* was 44.5 kDa. The crude OMP obtained by the above methods was mixed with loading buffer. The samples were then heated at 100 °C for 5 min to denature the proteins. Lee [13] reported that protein samples (10 µL) were loaded into the sample wells (3 % stacking gel) of the vertical electrophoresis tank at a stable 24 V (about 1 h). The voltage was changed to 75 V when the sample was close to the resolving gel (8 %). The resolving gel (stacking gel removed) was stained by Coomassie Brilliant Blue R-250 (CB B R-250) and destained with the destaining solution (methanol, acetic acid and distilled water; 4.5:1:4.5, v/v). The silver staining kit was also used to analyze the protein samples (Bio-Rad Laboratories Inc., Berkeley, USA).

Western Blot

To verify whether the protein samples prepared are immunological, we carried out western blot analysis with the semi-dry transfer electrophoresis tank recommended [14, 15]. The sample was observed by SDS-PAGE electrophoresis. We prepared six pieces of absorbent paper and a nitrocellulose membrane (NC membrane, 0.45 µm), the size of which was the same as that of the SDS-PAGE gel. Six layers of the paper and NC membrane were then put into transmembrane fluid [25 mM Tris-base, 0.2 M glycine, 20 % methanol (pH 8.3)] for 10 min, and three layers of the paper, the gel, NC membrane and another three layers (called the “sandwich”) were placed in order, air bubbles being removed with a glass rod. The “sandwich” was placed into a semi-dry electroblotting apparatus (Beijing sixty-one instrument) at 20 mA for 2.5 h. Subsequently, the NC membrane was slowly shaken at room temperature for 5 min in 25 mL TBS (0.242 % Tris-base, 0.8 % NaCl, pH 7.6 adjusted by HCl), and then transferred to 25 mL blocking solution (5 % skimmed milk powder in TBS) at 4 °C for 1–2 h or overnight. The membrane was washed three times (5 min/time) with 15 mL TBS/T (0.1 % Tween-20 in TBS) when its temperature reached room temperature. The NC membrane was put into the first antibody solution (20 mL 1:100 dilutions of whole-cell rabbit antiserum with TBS/T) and slowly shaken at 37 °C

for 1–2 h or 4 °C overnight. After washing it three times, the membrane was slowly shaken at 37 °C for 1 h in the second antibody solution [goat anti-rabbit IgG (HRP), 1:3,000 dilutions]. The membrane was then washed four times under the above conditions. Finally, the membrane was stained with the diaminobenzidine (DAB) substrate kit (Kang Wei Century Biotech Co., Ltd. Beijing, China) stored at 4 °C.

Results

Recommended Method, Sample and Centrifugation Time

Figure 1 shows the differences in the yields using the three sample preparation methods. Lane A (second method) shows that the sediment contained additional proteins besides the target protein. Compared with the other lanes, lane B (third method) indicated that this method gave the largest amount of the target proteins in the sediment, whereas the first method gave the lowest level (lane C). Additionally, the supernatant was shown to contain both the target proteins and other proteins when using the first method (lane D). Clearly, the third method is the best approach to obtain the OMP of *F. necrophorum (AB)*. We also compared samples of different time periods (method two) by SDS-PAGE. The results suggested that the supernatant in lane C contained more target proteins than that in lane B, and the sediment in lane A had a larger amount of target proteins (Fig. 2).

Fig. 1 Protein samples were prepared according to the extraction process methods of Zhu, Ainsworth and Takada. Lane A (sediment, 14,000 rpm, 4 °C, 3 h) followed by Zhu's extraction process, lane B (15,000 rpm, 4 °C, 3 h, this experiment), lane C (the sediment, 6,000 rpm, 4 °C, 30 min) and lane D (the supernatant, 6,000 rpm, 4 °C, 30 min) followed by Ainsworth's method

Fig. 2 Protein samples were prepared according to Zhu's extraction process (method two). *Lane A* (sediment, 14,000 rpm, 4 °C and 3 h), *lane B* (supernatant, 14,000 rpm, 4 °C, 3 h) and *lane C* (supernatant, 14,000 rpm, 4 °C, 30 min) were compared with *lane M* (marker)

Fig. 3 SDS-PAGE showing the presence of the OMP of *F. necrophorum* located in *lane A* (sediment, 15,000 rpm, 4 °C, 3 h) in this experiment, compared with the protein molecular weight markers, *lane M* (marker)

Fig. 4 *Lane A* was the sample (sediment, 15,000 rpm, 4 °C, 3 h) used by silver staining in this experiment. *Lane M* was the molecular weight markers (TaKaRa)

Fig. 5 Western blot with the polyclonal serum showing the sample (sediment, 15,000 rpm, 4 °C and 3 h) in this experiment (*lane A*) compared with pre-stained Marker (*lane M*)

The Molecular Weight of OMP and a Comparison of the Two Staining Methods

We chose lane M as the marker and used the third method to prepare samples. lane A shows that the molecular weight of the target protein was 44.5 kDa (Fig. 3). The silver staining also identified the existence of other proteins in the sample, but the brightest band represented the target protein (Fig. 4).

Western Blot

In addition, the results of the western blot analysis with a polyclonal serum suggested that the band of 44.5 kDa was still the strongest despite the identification of other proteins (Fig. 5). This method confirms the immune activity of the proteins.

Discussion

Due to the absence of an ultracentrifuge, this research aimed to modify methods [3, 10, 11] based on the available equipment in our laboratory. After 3 h of centrifugation at 15,000 rpm, the sample was identified by SDS-PAGE and stained by CB B R-250. The results showed that pure target protein could be obtained by the third method because SDS-PAGE confirmed the presence of only a single band that represented the OMP (Fig. 1). By changing the centrifugation speed and time, we also found that the sediment (14,000 rpm, 4 °C, 3 h) contained more target proteins (Fig. 2) when compared with the three sample preparation methods suggested by Zhu [10]. The target band was identified and stained by the above method, and the molecular weight of the protein was 44.5 kDa (Fig. 3).

We assumed that there should be many kinds of proteins that could be identified by CB B R-250 following the preliminary extraction, but none of them were found.

Initially, this method was thought to be better than before; however, the silver staining method was found to be more suitable to stain proteins because of its higher sensitivity compared with CB B R-250 [16], which resulted in the appearance of multi-bands in the lane (Fig. 4). The difference of the two results may be attributed to the negligible amounts of other proteins that could not be clearly identified by CB B R-250 [17] but only when using a silver staining technique. This indicates that the extraction of the OMP in this research tends to stay at the preliminary level. In addition, the condition (100,000 rpm, 4 °C and 1 h) cannot be achieved without a hypervelocity centrifuge in our lab. Therefore, the OMP dissolved with 2 % lithium dodecyl sulfate (LDS) in 20 mM HEPES-LiCl (pH 7.4) was modified following 3 h of centrifugation at 15,000 rpm at 4 °C. The change plays a key role in this method, because the extension of the centrifugation time may enhance the production of the target protein and assist in the successful extraction of the OMP. The adoption of western blot analysis demonstrated that there were additional proteins in the preliminary extraction (Fig. 5). This result suggests the immunological activity of the OMP and the possibility to purify the OMP (44.5 kDa) in the future. Many reports have shown that the OMP from various gram-negative bacteria has sensitive immune responses. Early research predicted that OMP may represent a new type of non-cell vaccine [18]. For example, Thavaselvam et al. [19] reported the cloning of OMP (Omp28) from *Brucella melitensis* and their evaluation of its expression products in the clinical diagnosis of brucellosis.

In conclusion, the method introduced in this report is useful for the preliminary extraction of the 44.5 kDa OMP of *F. necrophorum* (AB) with immunogenic activity. This research provides evidence for the application of OMP of *F. necrophorum* (AB) in the clinical setting.

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