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Short Communication

Preparative SDS-PAGE Electroelution for Rapid Purification of Alkyl Hydroperoxide Reductase from *Helicobacter pylori*

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Abstract

Background: Alkyl hydroperoxide reductase (AhpC) of *Helicobacter pylori* is considered as a diagnostic antigen. Therefore, this antigen can be used to detect *H. pylori* infection by stool immunoassays such as ELISA. The aim of this study was to simplify the AhpC protein purification procedures.

Methods: For whole cell protein extraction, the bacterial cells were ruptured by octly-β-D glucopyranoside. The isolation and purification of AhpC protein were attempted by various techniques including ammonium sulfate precipitation, dialysis, preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution.

Results: A simple method was used for protein purification AhpC protein. One-dimensional preparative gel electrophoresis allows a single and short purification step; the high resolution capacity of this technique leads to a high level of purity of the protein. Moreover, it avoids contamination by other non-specific proteins which often appear during protein purification by column chromatography.

Conclusion: The present method is simple, rapid and makes it possible to preparate AhpC from *H. pylori*.

Keywords: *Alkyl hydroperoxide reductase, AhpC, Electroelution,* Helicobacter pylori, *SDS-PAGE*

Introduction

Helicobacter pylori, an oxygen-sensitive microaerophilic bacterium, contains an alkyl hydroperoxide reductase homologue (AhpC) that is closer to eukaryotic peroxiredoxin than to other bacterial AhpC proteins (1). *H. pylori* AhpC is a major component of the AhpC-thioredoxin- thioredoxin reductase dependent peroxiredoxin system that catalyzes the reduction of hydroperoxides including H_2O_2 and organic hydroperoxides (1), and the reduction of peroxinitrite (2). The AhpC protein has previously been reported as species-specific protein which is antigenically conserved (3). Although not identified as a peroxidase at that time, the AhpC was characterized as a homodimer of 26 kDa polypeptide chains with inter-chain disulfide linkage and the protein was also suggested to be useful as a diagnostic antigen in enzyme immunoassay (EIA) tests for detection of *H. pylori* infection (3).

H. pylori express abundant levels of AhpC protein. Based on densitometric measurement of the protein bands on the gel, it has been shown that the protein constitutes more than 2% of the total protein in the wild- type cell (4) which confirms of the results of proteome analysis which has been shown AhpC as the third most abundant protein in *H. pylori* (5).

Results of another study has shown about 20-30% sequence homology between *H. pylori* AhpC and other bacterial AhpC, with as high as 43% sequence homology between the protein and the mammalian (human or mouse) peroxiredoxin (6). In addition, by immunoblotting the stool of the infected individuals, it has been shown that the 26 kDa protein antigen was present in all samples and has been suggested that this antigen is one of the major antigens of *H. pylori* which is released into the stool and can be considered as a diagnostic antigen which might be used in diag-

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nostic kit development (7). Furthermore, by applying comparative proteomic and immunoproteomic analysis of different *H. pylori* strains, AhpC has been described as a protein with potential diagnostic and therapeutic value (8).
AhpC is among the most conserved and unique *H. pylori* antigens. It may also serves as a potential target for antimicrobial agents or vaccine development (9).
In order to obtain the purified AhpC we used preparative SDS-PAGE and electroelution techniques. Although preparative SDS-PAGE and electroelution are well-described methods, they usually require appropriate apparatuses. Furthermore, this approach has not been previously reported for AhpC purification from *H. pylori*.

Materials and Methods

Bacterial strains

Five strains of *H. pylori* were isolated from biopsy specimens of 5 patients with gastritis. Biopsies were delivered to Laboratory of Microbiology, Faculty of Sciences, Tehran University, in transport medium. Samples were cultured immediately on selective *Brucella* agar containing 5% sheep blood, vancomycin (5 mg/l), trimethoprim (5 mg/l) and polymyxin B (2500 u/l). After 2-3 d of microaerobic incubation at 37 ^{o}C, single colonies were cultured on *Brucella* blood agar. Bacterial strains were identified as *H. pylori* according to microscopic observation of Gram-negative spiral bacteria and positive catalase, oxidase and urease reactions.
To obtain about 7 grams of a mixed bacterial pellet, 400 plated cultures of 5 *H. pylori* strains were harvested in to phosphate-buffered saline (PBS) and centrifuged at 5000 g for 20 min. Bacterial pellet was stored at -20 ^{o}C until use.

Protein extraction from bacteria

In order to extract proteins from *H. pylori*, frozen cell pellets were thawed, suspended in PBS (pH 7.2) containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) (Across Organics, New Jersy, USA), 4 mM EDTA and 0.6% (w/v) octyl-β-D glucopyranoside (Sigma-Aldrich, St. Louis, USA) and incubated for 60 min at the ambient temperature with gentle agitation. Samples were then centrifuged at 6000 g for 15 min and the supernatant was collected. The procedure was repeated once more on the bacterial pellet. The pooled supernatants were cleared by centrifugation at 35000 g for 20 min and 50% ammonium sulfate was added to the supernatant. After overnight incubation at 4 ^{o}C, the sample was spun at 35000 g for 30 min and the pellet was suspended in 5 ml of 0.05 M Tris-HCl containing 0.145 M NaCl (Tris-saline [pH 7.5]). Then the sample was dialyzed against the same buffer and protein assay was performed as described (10).

SDS-PAGE

One dimensional preparative and analytical SDS-PAGE were performed in a vertical slab gel unit using 12.5% separating gels and 5.0% stacking gels (11). The protein extract (1.5 ml) was separated by preparative SDS-PAGE (180×160×2.5 mm) using Tris-HCl buffer and stained with Coomassie Brilliant Blue solution [10% acetic acid, 40% methanol and 0.1% Coomassie Brilliant Blue (CBB)-R250]. Analytical electrophoresis was also done as described above.

Removal of CBB-R250 and electrophoretic elution of proteins from gel

In order to extract the protein from polyacrylamide gel, the method of electrophoretic elution was applied using dialysis membrane for protein retention (12-14).
Protein band with 26 kDa size was excised and cut into small fragments. Removing of CBB-R250 from the gel fragments was performed according to the described method (15). Briefly, destaining solution containing 50% isopropanol and 3.0% SDS was added to gel pieces in 12×75 mm glass test tubes and the tubes were capped with parafilm. Tubes were placed in a 37 ^{o}C water bath set for overnight without agitation. After cooling to room temperature, the liquid was removed and the gel fragments containing the appropriate protein were used for electrophoretic elution.

For electophoretic elution, gel fragments were equilibrated twice in 0.125 M Tris-HCl buffer (pH 6.8) and 2.0% solution of 2-mercaptoethanol for 15 min. A final equilibration of the gel fragments in 0.125 M Tris-HCl buffer (pH 6.8) containing 1.0% (w/v) SDS was performed. The equilibrated gel fragments were then placed in a dialysis tube with a minimum amount of tris-glycine buffer containing SDS (25 mM Tris, 192 mM glycine, and 0.1% SDS). The dialysis tubes were treated and electroelution was carried out as described by previously (14). Briefly, electroelution was performed at 50 V for 12 h at 4 ^{o}C in Tris-glycine buffer containing 0.1% SDS (pH 8.3). At the end of electrophoretic elution, the polarity of the electrodes was changed for 60 S in order to avoid the absorption of protein on the dialysis tubes.

Removal of SDS and renaturation of enzymatic activity

Remove of SDS and renaturation of enzymatic activity was performed as described (16). Briefly, four volume of cold acetone (-20 ^{o}C) was added to the electoeluted protein and the sample was allowed to precipitate 30 min in a dry ice-ethanol bath. The tube was then centrifuged 10 min at 10,000 rpm, the acetone supernatant poured off, and the tube was inverted to drain.

In order to renaturation of enzymatic activity, the acetone precipitate was allowed to dry for about 10 min and dissolved in 20 µl of 6 M guanidine-HCl in dilution buffer consisted of 0.05 M Tris-HCl, 20% glycerol, 0.1 mg/ml BSA, 0.15 M Na Cl, 1 mM dithiothreitol (DTT), and 0.1 mM EDTA. The pellet was dissolved thoroughly and allowed to stand at room temperature for 15-20 min. The solution was then diluted 50-fold with dilution buffer and permitted to renaturation overnight at room temperature.

AhpC enzymatic activity assay

The peroxide reductase activity of AhpC was monitored with a CECIL 9000 spectrophotometer (Cambridge, England) at 25 ^{o}C by following the decease in A_{340} within 12 min due to NADPH oxidation. Each assay was performed in a total volume of 1.0 ml of 50 mM potassium phosphate buffer (pH 7.0)/ 0.1 M ammonium sulfate/ 0.5 mm EDTA containing thioredoxin system, 0.5 µM thioredoxin, 0.5 µM thioredoxin reductase, 150 µM NADPH and 1 mM H_2O_2 (1,6).

Results

Assuming that the total protein content of the cells is 30% on a dry weight basis (17), then the expected maximum protein content in 0.92 g (dry weight) of bacterial cell lysate in a volume of 25 ml PBS, pH 7.4 would be 11 mg/ml, if all of the protein content is released. In practice, the total intracellular protein, 3.5 mg/ml, was determined by complete extraction of the protein from the cells with 4.0 M sodium hydroxide. The details of protein purification are given in Table 1.

The crude extract from *H. pylori* showed a brownish color and contained approximately 30 bands when analyzed in a CBB-R250 stained SDS-PAGE gel as shown in Fig.1, lane A. Fig. 1, lane B shows that in an analytical SDS-PAGE, the electroeluted protein migrated as a single band confirming its purity to homogeneity.

Fig. 2 shows the various steps and a summary of the techniques used in the present work to purify the AhpC protein antigen to homogeneity.

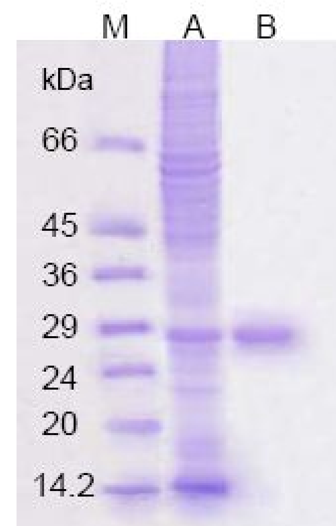

Fig. 1: Protein profile of *H. pylori*. Lane M: molecular weight standard, Lane A: whole cell protein extract, Lane B: purified AhpC

Table1: Details of AhpC protein purification from *H. pylori* and evaluation of its enzymatic activity

Purification step	Protein (mg)	Total activity (U)	Specific Activity (U/mg protein)	Yield (%)	Fold purification
Crude homogenate (50 ml)	150	26210	174	100	1
35000 ×g supernatant (43 ml)	95	24797	261	95	1.5
$(NH_4)_2$ SO_4 fraction (0-50%)(5 ml)	70	23142	330	88	1.9
After dialysis (6 ml)	44	21437	487	82	2.8
Electroelution (3ml)	0.9	15726	17473	60	100.4

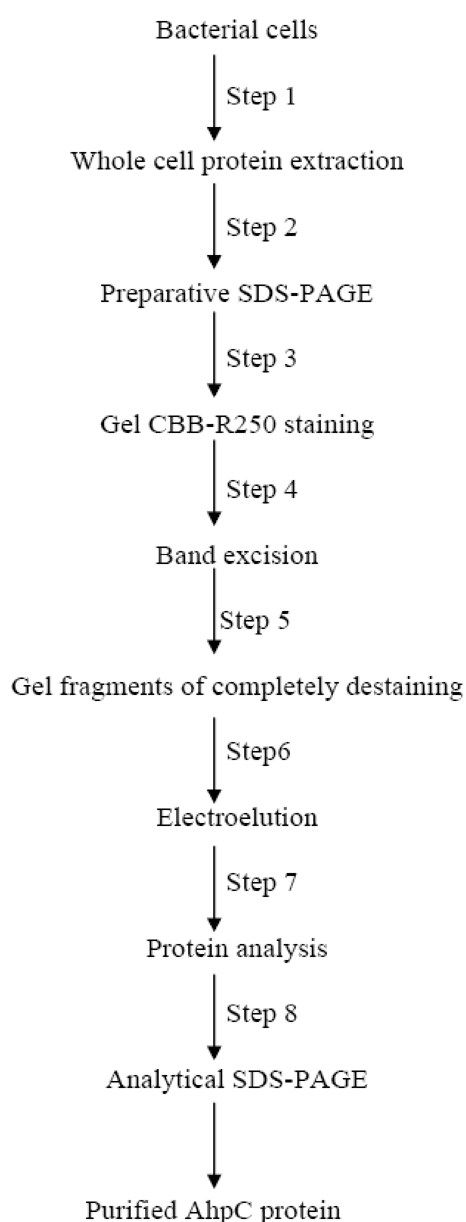

Fig. 2. Flow chart showing the purification procedure of *H. pylori* AhpC protein.

Discussion

There has been a growing interest in stool antigen tests for detection of *H. pylori*. The application of polyclonal antibodies against *H. pylori*-specific antigens such as AhpC may increase the specificity of the EIA. Furthermore, AhpC protein has been suggested as a potential target for the development of therapeutic agents against *H. pylori* and anti-AhpC is useful for proteomic studies of *H. pylori*.

H. pylori AhpC protein is typically purified using two or more chromatographic steps (3, 18-19). These protocols often involve a precipitation step, followed by an ion-exchange and/or gel filtration chromatography. Another approach is based on molecular biology tools, which involves cloning the codifying gene in order to produce the recombinant enzyme in a proper expression vector (1, 6, 20).

In the present work, a different methodology was used to simplify the purification of *H. pylori* AhpC protein. This approach is based on preparative SDS-PAGE and electroelution.

A summary of the techniques used in the present work to purify the AhpC protein has been shown in Fig. 2. The bacterial cells were first disintegrated by 0.6% octly β-D-glucopyranoside as non-ionic detergent. Then, the whole cell protein extraction was carried out by various techniques including ultracentrifugation, ammonium sulfate precipitation and dialysis techniques (Fig. 2, step 1). At the end of protein extraction, the concentration and specific activity were determined to be 7.4 mg/ml and 487 U/mg protein respectively (Table 1). For the preparative SDS-PAGE, approximately four milligram of protein extract was run for about 12 h at 50 V (Fig.2, step 2). The molecular weight standard was applied in the middle well between two wide wells to determine the approximate size of protein bands. After electrophoresis, the gel was stained with CBB-R250 and destained for revealing the protein bands (Fig. 2, step 3) and the 26 kDa protein band was excised precisely, cut into pieces, (Fig. 2, step 4), and destained completely according to the described method (15) (Fig .2, step 5). Gel pieces were subsequently dialyzed in bags previously filled with 25 mM Tris buffer containing 192 mM glycine and 0.1% SDS, pH 8.3. The appropriately identified tubing was placed in a horizontal flat bed gel electrophoresis apparatus, filled with the same buffer mentioned above. Electroelution of the protein from gel fragments was performed at 50 V (Fig. 2, step 6) for 12 h. Finally, the gel fragments were discarded and the protein concentration was determined to be 300 μg per milliliter. In addition to, after remove of SDS and renaturation of enzymatic activity the Ahpc activity was measured to be 17473 U/mg protein (Table 1). In fact, analysing the specific activity of the electroeluted fraction of purification process revealed that the Ahpc was purified more than 100-fold from whole cell lysate (Table 1). Fig. 1, lane B shows that in an analytical SDS-PAGE, the electroeluted protein migrated as a single band confirming its purity to homogeneity (Fig. 2, step 8).

While the AhpC protein of *H. pylori* has been purified by several multi-step procedures (3, 18-19) the present report provides a simple method of purification. Moreover, several types of apparatus for electroelution are commercially available (21), but we used only electrophoretic elution using dialysis membrane for protein retention with good results.

In the present study, we used a simple method to purify AhpC which avoids the long term purification of the AhpC protein. One-dimensional preparative gel electrophoresis allows a single short purification step and the high resolution of this technique leads to a high level of protein purity. Moreover, it avoids contamination by other non-specific proteins which often appear during protein purification by column chromatographic techniques. In conclusion, the present method is simple, rapid and makes it possible to preparation AhpC from *H. pylori*.

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