Production of monoclonal antibody against alkylhydroperoxide reductase (HP 1563) of Helicobacter *pylori* and its antigenicity

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ABSTRACT

Development of a stool antigen immunoassay to detect *Helicobacter pylori* infection requires monoclonal antibody against the specific antigen. Alkylhydroperoxide reductase (AhpC) of *Helicobacter pylori* has been described as a specific and unique enzyme for *H. pylori* and therefore, both *H. pylori* AhpC and Anti-AhpC could be useful in the development of serologic and stool antigen tests, for detecting and monitoring *H. pylori* infection. The aims of this study were to prepare a monoclonal antibody against AhpC. Accordingly, The isolation and purification of AhpC from *H. pylori* were attempted by various techniques including ammonium sulfate precipitation, dialysis, preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroelution. Furthermore mice were immunized intraperitoneally with homogenized gel containing the AhpC band of protein extract of *H. pylori* in sodium dodecyl sulfate- polyacrylamide gel electrophoresis. The monoclonal antibody was produced using the hybridoma technique.
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 enzyme and consequently to a very high specificity of the antibody. The high specificity of antibody was identified by immune blotting in which the antibody reacted with the purified AhpC and whole cell protein extract from *H. pylori* in addition to the intact cells of *H. pylori*. This approach is simple, time and cost-saving for preparation of monoclonal antibody against AhpC of *H. pylori*.

Keywords: Alkylhydroperoxide reductase; *Helicobacter pylori*; Peroxiredoxin

INTRODUCTION

Helicobacter pylori, a spiral Gram-negative microaerophilic bacterium, contains an alkylhydroperoxide reductase (AhpC) homologue that is closer to eukaryotic peroxiredoxin than other bacterial AhpC [1, 2]. *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, and the reduction of peroxinitrite [1-3]. In addition to AhpC, there are two other members of peroxiredoxin family in *H. pylori* which are called thiol-specific peroxidase (Tpx), and bacterioferritin comigratory protein (Bcp) that have similar activities as the AhpC [4-6].
The AhpC has previously been reported as a species-specific protein that is antigenically conserved [7]. Although not identified as a peroxidase at that time, the alkylhydroperoxide reductase was characterized as a homodimer of 26 kDa polypeptide chains with inter-chain disulfide linkages and the protein was also suggested to be useful as a diagnostic antigen in enzyme immunoassay (EIA) tests for detection of *H. pylori* infection [7].
H. pylori express abundant levels of AhpC. Based on densitometric measurement of the protein bands on the gel, it has shown that this protein constitutes more than 2% of the total protein in the wild- type cell [8], confirming the results of proteome analysis which showed AhpC is the third most abundant protein in *H. pylori* [9]. Sequencing analysis revealed 20-30% homology between *H. pylori* AhpC and other bacterial AhpC, and around 43% homology with mammalian peroxiredoxin [10]. In addition, by immunoblotting on the stool of the infected

individuals, Pourakbari et al. showed that the AhpC antigen was present in many samples and 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 to detect *H. pylori* infection [11]. In Mohammadian et al reports using Western blot and immunodot blot analyses they also showed that AhpC subunits are detectable in the fecal antigenic extract of infected patients [12, 13]. Furthermore, using comparative proteomic and immunoproteomic analysis of different *H. pylori* strains revealed AhpC as a protein with potential diagnostic and therapeutic values [14].

Alkylhydroperoxide reductase is one of the most conserved and unique *H. pylori* antigens. Thus, antibodies against this protein are potentially useful tools for detecting and monitoring *H. pylori* infection in stool. It may also serve as a potential target for antimicrobial agents or vaccine development [15]. In addition, The AhpC has been identified as biomarker related to *H. pylori* associated gastro duodenal disease [16]. In the present study we tried to produce a monoclonal antibody against AhpC, in order to detect *H. pylori* infection by enzyme immunoassay.

MATERIALS AND METHODS

Bacterial strains

Five clinical strains of *H. pylori* were isolated from biopsy specimens of five patients with gastritis. Biopsies were delivered to the microbiology laboratory 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 days 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 6.5 g of a mixed bacterial pellet, 400 plated cultures of five *H. pylori* strains were harvested and suspended in phosphate-buffered saline (PBS) and centrifuged at 5000×g for 20 minutes. 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 mmol/L phenylmethylsulfonyl fluoride (Acros Organics, Fair Lawn, NJ, USA), 4 mmol/L EDTA and 0.6% (w/v) octyl-β-D glucopyranoside (Sigma-Aldrich, St Louis, MO, USA), and incubated for 60 minutes at an ambient temperature with gentle agitation. Samples were then centrifuged at 6000×g for 15 minutes and the supernatants were collected. The procedure was repeated once more on the bacterial pellet. Pooled supernatants were cleared by centrifugation at 35,000×g for 20 minutes and 50% ammonium sulfate was added to the supernatant. Following overnight incubation at 4 ^{o}C, the sample was spun at 35,000×g for 30 minutes and the pellet was suspended in 5 mL of 0.05 mol/L Tris-HCl containing 0.145 mol/L NaCl (Tris-saline [pH 7.5]). The sample was dialyzed against the same buffer and protein assay was performed as described previously [17].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

One-dimensional preparative and analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAG) were performed in a vertical slab gel unit using 12.5% separating gels and 5.0% stacking gels [18]. The protein extract (1.5 mL) was separated by preparative SDS-PAGE (180 ×160 × 2.5 mm) using Tris-HCl buffer (pH 8.8) and stained with Coomassie Brilliant Blue solution [10% acetic acid, 40% methanol and 0.1% Coomassie Brilliant Blue (CBB)-R250]. Analytical electrophoresis was also performed as described above.

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

In order to extract the protein from polyacryl amide gel, a method of electrophoretic elution was applied using dialysis membrane for protein retention [19-23]. 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 Ball method [23]. 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 mouse immunization and electrophoretic elution. For electophoretic elution, gel fragments were equilibrated twice in 0.125 mol/L Tris-HCl buffer (pH 6.8) and 2.0% solution of 2-mercaptoethanol for 15 minutes. A final equilibration of the gel fragments in 0.125 mol/L 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 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS). The dialysis tubes were treated, and electroelution was carried out as described previously [20]. Briefly, electroelution was performed at 50 V for 12 hours 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 seconds in order to avoid the absorption of protein on the dialysis tubes.

Production of monoclonal antibody

BABL/c mice were immunized with homogenized gel fragments containing approximately 20 µg of the protein without Freund's adjuvant by intraperitoneal injection and boosted at 3 weekly intervals with a further 20 µg of 26 kDa protein, a total of four injection. Monoclonal antibody production was carried out essentially by the method Köhler and Milstein (24). Exception that cell fusion was achieved using polyethylene glycol. Monoclonal antibody fusion supernatants were tested against a crude antigen preparation of *H. pylori* in ELISA and immunoblotting. A hybridoma secreting the appropriate monoclonal was recloned a second time to preserve stability.

Indirect enzyme-linked immunosorbent assay

Titration of the antibodies in the supernatant of clones were performed by indirect enzyme-linked immunosorbent assay (ELISA), using the purified 26 kDa protein as the antigen.

ELISA wells were coated with 100 µL of 26 kDa protein (0.2-1 µg/mL) in PBS (pH 7.5) and incubated at 4^{o}C for overnight. After incubation, plates were washed three times with washing solution [PBS containing 0.05% Tween 20 (PBS-T)] and nonspecific sites were blocked with 2% bovine serum albumin (BSA) in washing solution for 2 hours at 37 ^{o}C. After blocking, plates were washed as done previously and incubated with hybridoma suprernatant for 2 hours at 37 ^{o}C. The plates were washed and incubated with goat anti-mouse IgG-peroxidase conjugate for 2 hours at 37^{o}C; then unbound conjugated antibodies were removed by washing and color development was performed using 3, 3', 5, 5' tetramethyl benzidine (TMB) as substrate. Finally the reaction was stopped with 50 µL per well of 1 mol/L HCl, and the absorbance at 450 nm was recorded using microplate reader (Anthos Labtec, Salzburg, Austria).

Western immunoblotting

Immunoblotting was performed as described previously [25]. Briefly, *H. pylori* extracts were electrophoresed on SDS-PAGE gel and separated proteins were blotted on nitrocellulose paper (NCP). After blotting, NCP was blocked with 2% solution of BSA in PBS-T for 1 hour at room temperature. The NCP was then incubated with the appropriate dilution (1:20) of antibody in PBS-T for 2 hours. The NCP was then washed three times with PBS-T and goat antirabbits IgG conjugated to horseradish peroxidase (HRP) (1:500) was added and incubated for 1hour at room temperature. After incubation, the NCP was washed three times with PBS-T, and protein bands were visualized by adding a mixture of hydrogen peroxide as the HRP substrate and 3,3′-diamino tetra hydrochloride (DAB) (BHD limited, Poole UK) as the chromogen reagent.

Immunodot blotting

Dot blotting was performed by transferring 10 µg of protein sample to NCP. After the NCP was dried at 37 ^{o}C for 1 hour, it was blocked with a 2% solution of BSA in PBS-T. After blocking, the appropriate dilution of antibody was added to NCP and incubated at room temperature for 1 hour. Finally, NCP was washed and developed by the procedure described for Western blotting.

RESULTS

In the first step, cultured bacterial clinical strains were identified as *H. pylori* according to microscopic observation of Gram-negative spiral bacteria and positive catalase, oxidase, and urease reaction (Figure 1). Figure 2 shows preparative SDS-PAGE of whole cell protein extract of *H. pylori*. The molecular weight standard was applied in the middle well between two wide

wells to determine the approximate size of protein bands.

The crude extract from *H. pylori* showed a brownish color and contained approximately 30 bands when analyzed in a CBB-R 250 stained SDS-PAGE gel as shown in Fig 3, lane A. Figure 3, lane B shows that in an analytical SDS-PAGE, the electroeluted protein migrated as a single band indicating its purity to homogeneity.

In order to characterization of antibody, immunoblotting was performed. Fig. 4 and 5 show dot blot assay and Western blotting with antibody respectively.

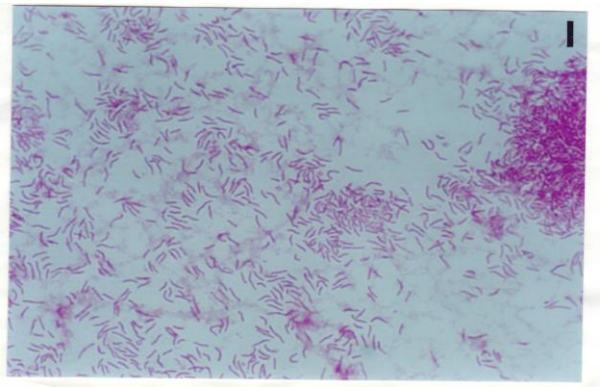

Figure 1. Microscopic view of *H. pylori* clinical strains. Gram-staining 1000X.

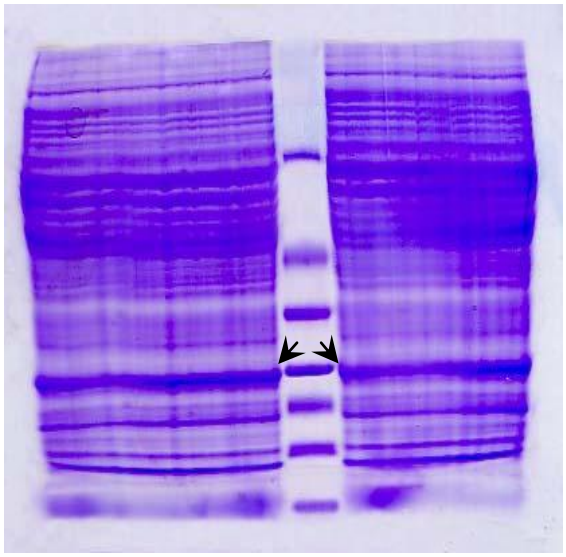

Figure 2. Preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *H. pylori* protein extract. The middle lane shows the molecular weight markers of 66, 45, 36, 29, 24, 20, and 14.2 kDa, respectively. Arrows indicate the 26 kDa position.

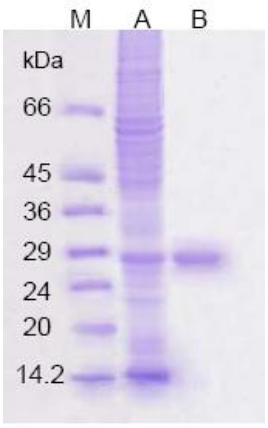

Figure 3. Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *H. pylori* extract and its purified alkylhydroperoxide reductase (AhpC). Lane M: molecular weight markers: Lane A: whole cell protein extract: Lane B: purified AhpC.

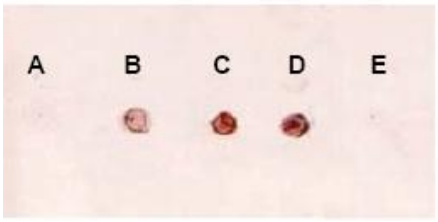

Figure 4. Dot blot assay with antibody. (A) cell extract from cultured *Escherichia coli* as negative control; (B) the purified 26 kDa; (C) whole cell protein extract; (D) intact *H. pylori* cells; (E) the purified thiol peroxidase (Tpx).

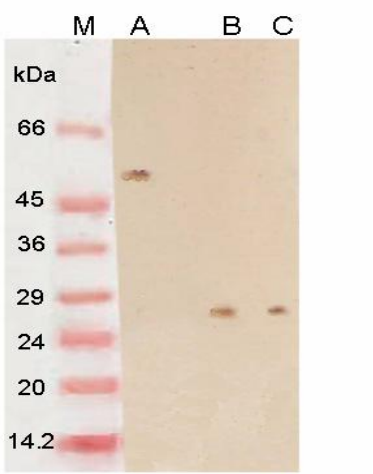

Figure 5. Western blot analysis using produced monoclonal antibody in mouse. Lane M: prestained protein markers, lane A: whole cell protein extract in non-reducing condition, lane B: whole cell protein extract in reducing condition, lane C: the purified 26 kDa protein (AhpC).

DISCUSSION

Stool-antigen detection kits for diagnosis of *Helicobacter pylori* infection have been widely used because of their full non-invasive nature. Because *Helicobacter pylori* strains show a distinctive genetic diversity, it is important to find a protein that is a common antigen of various strains and shows a strong immunogenicity for the development of a stool antigen detection kit.

Alkylhydroperoxide reductase of *H. pylori* is the most extensively studied *H. pylori* peroxiredoxin system. AhpC of *H. pylori* has been suggested to be a potential target for the development of therapeutic agent against *H. pylori* [15]. Furthermore application of monoclonal antibodies against *H. pylori*- specific antigens such as AhpC may increase the specificity of EIA for detecting and monitoring *H. pylori* infection, and anti-AhpC antibodies are useful tools for proteomic studies of *H. pylori*.

Alkylhydroperoxide reductase of *H. pylori* is typically purified using two or more chromatographic steps [7, 26-28]. 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, involving cloning the gene in an appropriate expression vector to produce the recombinant enzyme [1, 2, 10, 11, 29 and 30].

Furthermore, the way to produce monoclonal antibody against a protein is inject the protein into laboratory animals. The antigen can be obtained from standard purification techniques described above. However, the procedures are work consuming and costly.

In the present work, a different methodology was used to simplify the purification and antibody production against *H. pylori* proteins. This approach is based on preparative SDS-PAGE, electroelution and antibody production using the natural protein separated by gel electrophoresis.

In the first step, cultured bacterial clinical strains were as were identified as *H. pylori* according to microscopic observation of Gram-negative spiral bacteria and positive catalase, oxidase, and urease reaction (Figure 1).

The whole cell protein extraction was then carried out by various techniques including ultracentrifugation, ammonium sulfate precipitation, and dialysis techniques. For the preparative SDS-PAGE, protein extract was run at 50 V for 12 hours. 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 protein bands (Figure 2). The 26 kDa protein band was then excised, cut into small species and destained completely according to the Ball method [23]. Gel pieces were subsequently homogenized for mice immunization and dialyzed in bags previously filled with 25 mmol/L Tris buffer containing 192 mmol/L glycine and 0.1% SDS, pH 8.3. The appropriate 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 for 12 hours finally, the gel fragments were discarded and the protein concentration was determined to be 200 µg/mL with the enzymatic activity of 26210 U/mg protein. Figure 3 (lane B) shows that in an analytical SDS-PAGE, the electroeluted protein migrated as a single band indicating its purity to homogeneity.

While the AhpC of *H. pylori* has been purified by several multi-step procedures [7, 26-28] the present report provides a simple method for purification of this protein. Moreover, several type of apparatus for electroelution are commercially available [31, 32], but we used only electrophoretic elution using dialysis membrane for protein retention with good results. Furthermore, the protein band separated on a preparative SDS-PAGE elicited good immune response in mice after immunization with 26 kDa protein in gel, as measured by ELISA against a whole cell protein extract, because polyacrylamide helps to retain the antigen in the injection site, and thus acts as an adjuvant [33].

The specificity of the antibody was further identified by immunoblotting systems. As it is obvious in Figure 4 the antibody react with the same purified electroeluted antigen, whole cell protein extract of *H. pylori* and the washed intact bacterial cells in dot blot assay. The reaction with intact cells indicates that some part of AhpC is exposed on the bacterial cell surface, confirming the report by of Lindholm and coworkers, in which they showed that AhpC could be detected

on the surface of *H. pylori* [27]. It was also noted that our preparation of purified *H. pylori* thiol-specific peroxidase did not react with the antibody (Figure 4, dot E) which is in agreement with the results of Wan et al. [34]. Western blotting analysis revealed that the antibody can bind specifically with the dimeric native and denatured forms of the AhpC (Figure 5). Taken together, our results suggest that the antibody can specifically detect *H. pylori* AhpC.

In the present study, we used a simple method to purify AhpC and produce antibody against it. This technique avoids both the long term purification of the AhpC and the need to add Freund's adjuvant.

CONCLUSION

The approach presented here has following advantages: it does not require special expensive apparatus and reagents and preparative-scale amounts of AhpC could be purified. It is not time consuming and monoclonal antibody was produced against natural AhpC in gel bands instead of using recombinant protein for production monoclonal antibody.

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