A simple and cost-effective method for rapid purification of alkyl hydroperoxide reductase (AhpC) from *Helicobacter pylori* and its antibody production

[1]Mohammadian T., [*1]Doosti M., [1]Paknejad M., [2]Siavoshi F., [3]Massarrat S.

[1]Department of Medical Biochemistry, School of Medicine, Tehran University of Medical Sciences, [2]Department of Microbiology, School of Sciences, University of Tehran, [3]Digestive Disease Research Center, Tehran University of Medical Sciences,, Tehran, Iran.

Received 21 Apr 2008; Revised 18 July 2008; Accepted 21 July 2008

ABSTRACT

Background and the purpose of the study: Helicobacter pylori express abundant amounts of AhpC enzyme that functions to reduce organic hydroperoxides (ROOH) into the corresponding non-toxic alcohols (ROH). This conserved antigen has been earlier described as specific and unique for *H. pylori* and therefore, both *H. pylori* AhpC and Anti-AhpC could be useful in the development of serologic and stool antigen tests, to detecting and monitoring *H. pylori* infection. AhpC may also serves as a potential target for an antimicrobial agent or for vaccine development. The aim of this study was to simplify isolation and purification of the AhpC and production of a highly specific polyclonal antibody against it.

Methods and Results: In this paper a simple method was used for protein purification and antibody production which avoids both the long term AhpC protein purification procedure and the addition of Freund's adjuvant. One-dimensional preparative gel electrophoresis allows a single and short purification step and the high resolution capacity of this technique leads to a high level of purity of the protein and consequently to a very high specificity of the antibody. Moreover, it avoids contamination by other non-specific proteins which often appear during protein purification by column chromatographic techniques.

Major Conclusion: The present method is simple, rapid and cost-effective and makes it possible to produce antibody for stool antigen enzyme immunoassay in short time and at low cost.

Keywords: Alkyl hydroperoxide reductase; AhpC; Electroelution; *Helicobacter pylori*; Polyclonal antibody; Protein purification.

INTRODUCTION

Helicobacter pylori (*H. 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). In addition to AhpC protein, there are two other members of thioredoxin dependent peroxidase in *H. pylori* which are called thiol-specific peroxidase (Tpx), and bacterioferritin comigratory protein (BCP) that have similar activities as the AhpC protein (3).

The AhpC protein has previously been reported as species-specific protein which is antigenically conserved (4). 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 (4).

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 (5) which confirms results of proteome analysis indicating AhpC as the third most abundant protein in *H. pylori* (6).

Results of another study has shown about 20-30%

Correspondence: dtm136@yahoo.com

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 (7). In addition, by immunoblotting of the stool of the infected individuals, it has been shown that the 26 kDa protein antigen was present in all samples and it 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 diagnostic kit development (8). 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 values (9).

Since AhpC is among the most conserved and unique *H. pylori* antigens, development of antibodies against it is potentially useful for the stool antigen tests in order to detect and monitor *H. pylori* infection. It may also serves as a potential target for antimicrobial agent or vaccine development (10).

In the present study AhpC from *H. pylori* was purified and a polyclonal antibody against it was produced.

MATERIALS AND METHODS

Materials and instruments

Octyl- β-D- glucopyranoside, Bovine serum albumin (BSA), vancomycin, trimethoprim and polymyxin B were all purchased from Sigma-Aldrich St. Louis, USA. Brucella agar was obtained from Merck, Germany and phenylmethylsulphonyl fluoride (PMSF) was purchased from Across Organic, USA. 3, 3´- diamino tetra hydrochloride was purchased from BHD Limited Poole, England.

Absorbance was determined with a CECIL 9000 spectrophotometer (Cambridge, England). Absorbance of ELISA wells was recorded using an Anthos 2020 microplate reader (Anthos Labtec instrument Austria). Electroelution was performed using horizontal flat bed mini-gel electrophoresis apparatus (Pouya Pajouhesh, Mashhad, Iran). One dimensional preparative and analytical SDS-PAGE were performed in a vertical slab gel unit (Akhtarian, Tehran, Iran).

Bacterial isolation and cultivation

The *H. pylori* strains used in this study were isolated from biopsies of the stomach of patients with a variety of gastrointestinal disorders. The specimens were transported to the laboratory in sterile semisolid saline containing 0.16% agar and cultured on selective Brucella agar containing defibrinated blood (7%), vancomycin (5 mg/l) trimethoprim (5 mg/l) and polymyxin B (2500 u/l). After microaerobic incubation at 37 ^{o}C, bacterial cultures were purified as *H. pylori* according to microscopic observation of spiral morphology and catalase, oxidase, and urease positive reactions. Bacterial cells from culture plates were harvested in phosphate–buffered saline (PBS), centrifuged at 5000 g for 20 min, and the pellet was stored at -20 ^{o}C until use.

Whole cell protein extraction

For whole cell protein extraction, frozen cell pellets were thawed, suspended in PBS (pH 7.2) containing 1.0 mM PMSF, 4 mM EDTA and 0.6% (w/v) octyl-β-D-glucopyranoside. Extraction of proteins was allowed to take place for 60 min at the ambient temperature with gentle agitation. Cells were removed by centrifugation at 6000 g for 15 min and then re-extracted. The pooled supernatants were cleared by centrifugation at 35000 g for 20 min and ammonium sulfate was added to 50% saturation. 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-hydrochloride containing 0.145 M NaCl (Tris-saline [pH 7.5]). The sample was dialyzed against the same buffer. The protein assay was then performed according to the described method (11).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (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 (12). The protein extract (1.5 ml) was separated by preparative SDS-PAGE (180 ×160 × 2.5 mm) and stained with 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 slices

In order to extract the protein from SDS-PAGE, the method of electrophoretic elution was applied using dialysis membrane for protein retention (13-15).

Protein band with 26 kDa size was excised and cut into small fragments. Removal of CBB-R250 from the gel fragments was performed according to the described method [16]. Briefly, destaining solution containing 50% isopropanol and 3.0% SDS was added to gel pieces in 12×75 mm glass test tubes which were then capped with parafilm. Tubes without agitation were placed overnight at 37 ^{o}C water bath set. After cooling to room temperature, the liquid was removed and the gel

fragments containing the appropriate protein were used for rabbit immunization and electrophoretic elution.

For electophoretic elution, the 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 was carried out in 0.125 M Tris-HCl buffer (pH 6.8) containing 1.0% (w/v) SDS. The equilibrated gel fragments were then placed in a dialysis tube with a minimum amount of buffer containing SDS (25 mM Tris, 192 mM glycine and 0.1% SDS), and electroelution was carried out according to the reported method [15]. In this study, electroelution was performed at 50 V for 12 h at 4 ^{o}C in a buffer containing 25 mM Tris, 192 mM glycine and 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.

Immunization of rabbits

Polyclonal antiserum was raised in adult New Zealand white rabbits by intradermal and intramuscular injections. The rabbits were immunized with the homogenized gel fragments containing approximately 50 µg of the protein without Freund's adjuvant. Booster doses were also given without Freund's adjuvant on the days of 14 and 28. On the day of 42 the rabbits were exsanguinated, and the serum was collected and stored at -20 ^{o}C. Control preimmune serum was obtained before the first injection.

Titration of antiserum by enzyme-linked immunosorbent assay (ELISA)

The titer of antiserum was determined by means of indirect ELISA, in which the purified 26 kDa protein served as the antigen and the preimmune serum was used as the negative control.

A microtiter plate was coated with the 26 kDa protein at 37 ^{o}C overnight in PBS buffer (pH 7.5). Nonspecific binding was blocked with 2% BSA in PBS (pH 7.5) for 2 h at 37 ^{o}C and the plate was washed three times with PBS. The plate was then incubated for 2 h at 37 ^{o}C with antiserum. After washing, the plate was incubated with anti-rabbit IgG-Horseradish peroxidase conjugate for 2 h at 37 ^{o}C. Finally, after washing with PBS as before, color was developed by using 3,3',5,5' tetramethyl benzidine (TMB) as substrate. Finally the reaction was stopped with 50 ml/well of 1 M HCl, and the absorbance at 450 nm was recorded using ELISA reader.

Western immunoblotting

Immunoblotting was performed as described previously [17]. Briefly, After electroblotting, unreacted sites on the nitrocellulose paper (NCP) were blocked with a 2% solution of BSA in PBS (pH 7.5) for 1 h at room temperature .The NCP was then incubated with the appropriate dilution (1:20) of antiserum in the same buffer for 2 h. The NCP was washed three times with PBS. Goat anti-rabbits IgG conjugated to Horseradish peroxidase (HRP) was then added and incubated for 1h at room temperature. After incubation, the NCP was washed three times with PBS. The reaction bands were visualized with hydrogen peroxide as the HRP substrate and 3, 3′-diamino tetra hydrochloride (DAB) as the color development 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 h, it was blocked with a 2% solution of BSA in PBS (pH 7.5), treated with antiserum, washed and developed by the procedure which was described for western blotting.

RESULTS

Assuming that the total protein content of the cells is 30% on a dry weight basis (18), then the expected maximum protein content in 0.92 g (dry weight) of bacterial cell lysate in a volume of 25 ml PBS, of pH 7.4 would be 11 mg/ml, if all of the protein content is released. In practice, the total intracellular protein was determined 3.5 mg/ml, 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.

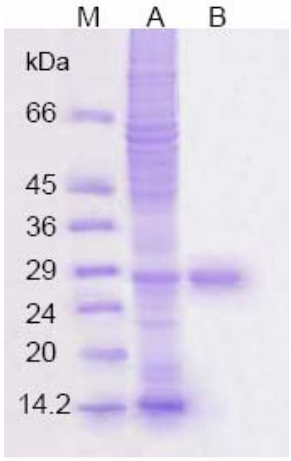

Figure 1. Protein profile of *H. pylori*. Lane M: molecular weight standard, Lane A: whole cell protein extract, Lane B: purified AhpC.

Table 1. Details of protein purification

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Recovery (% total protein)
Crude homogenate	50	3	150	100
35000 ×g supernatant	43	2.2	95	63
$(NH_4)_2 SO_4$ fraction (0-50%)	5	14	70	74
After dialysis	6	7.4	44.5	63
Electroelution	3	0.3	0.9	2

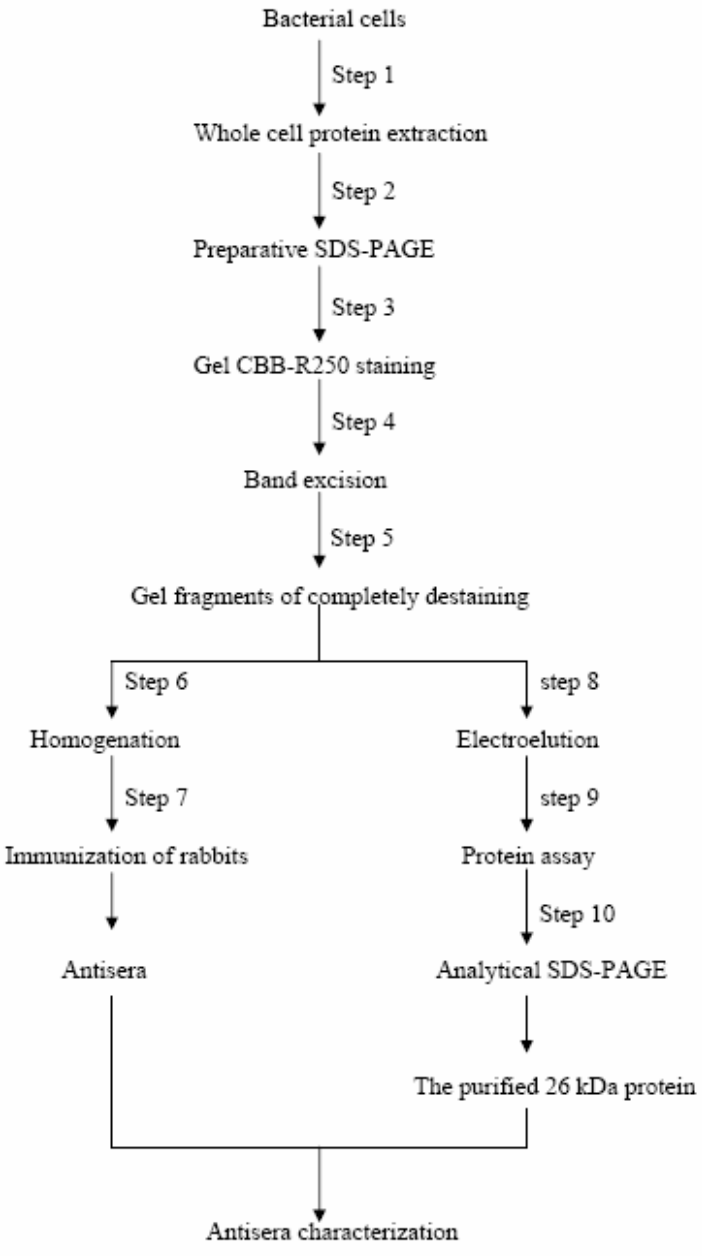

Figure 2. Flow chart showing the purification procedure and antibody production against the *H.pylori* AhpC protein.

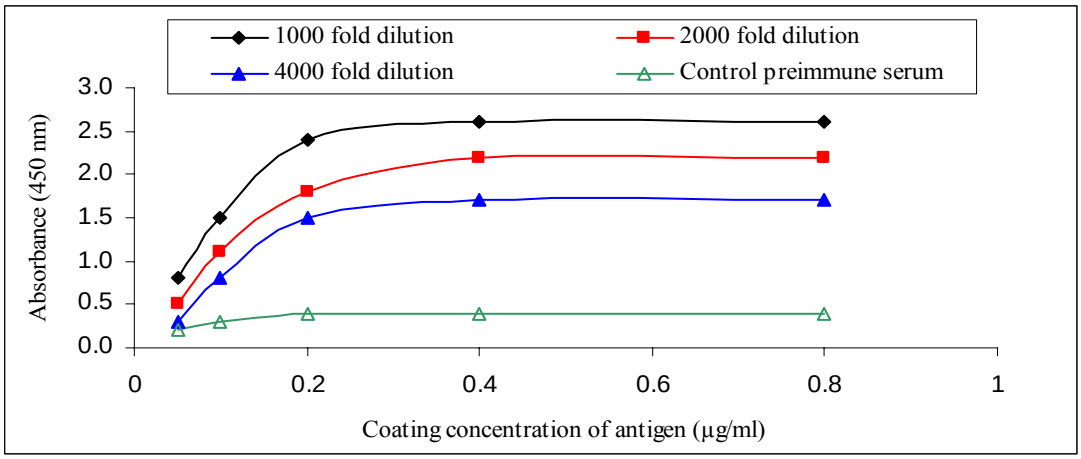

Figure 3. Binding of variable concentration of antibodies to a variable concentration of antigen.

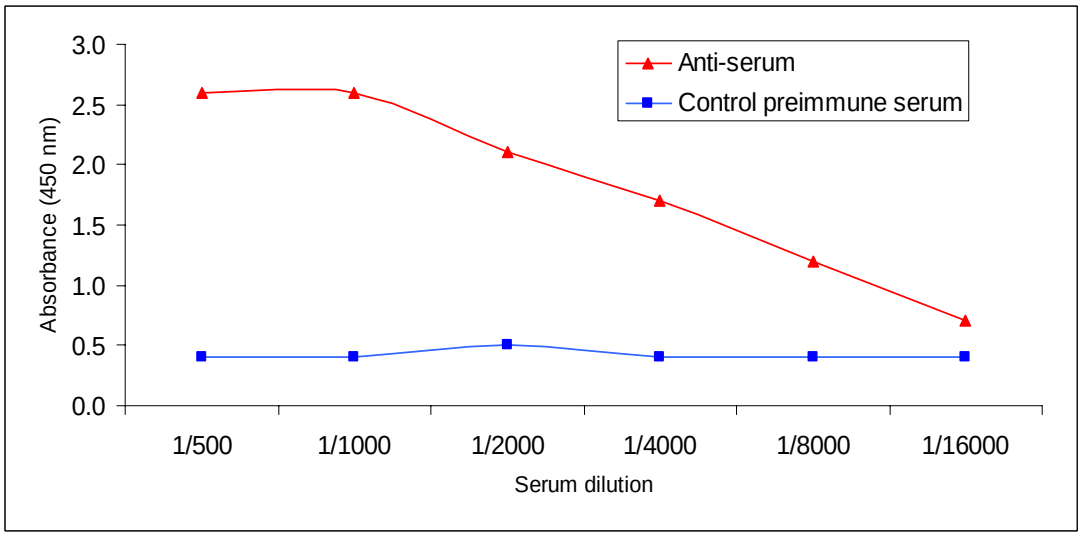

Figure 4. The titer of the anti-AhpC serum was analyzed by ELISA.

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 various steps and a summary of the techniques used in the present work to purify the AhpC protein antigen to homogeneity and preparation of protein gel band for the purpose of immunization of the rabbits, as well as antiserum characterization. Fig. 3 shows indirect ELISA assay to determine the proper concentration of purified AhpC for coating in ELISA wells. Titration of the anti-AhpC serum by ELISA assay has been shown in Fig.4.

In order to characterization of antiserum, immunoblotting was performed. Fig. 5 and 6 show dot blot assay and western blotting with antiserum repectively.

DISCUSSION AND CONCLUSION

There has been a growing interest in stool antigen tests for detection of *H. pylori*. Many studies show that the Premium Platinum HpSA EIA (Meridian Diagnostics, Cincinnati, OH, USA), which uses a nonspecific polyclonal antibody as capture reagent, has lower accuracy compared to monoclonal stool antigen tests (FemtoLab *H. pylori*, Connex, GmbH. Germany) (19-21).

The application of high specific polyclonal antibody against *H. pylori*- specific antigens such as the AhpC may increase the specificity of the EIA. Furthermore, the anti-AhpC is useful for proteomic studies of *H. pylori*. AhpC has been suggested as a potential target for the development of therapeutic agent against *H. pylori*.

The *H. pylori* AhpC protein is typically purified by two or more chromatographic steps (4, 22-23).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 an adequate expression vector (1, 7, 24).

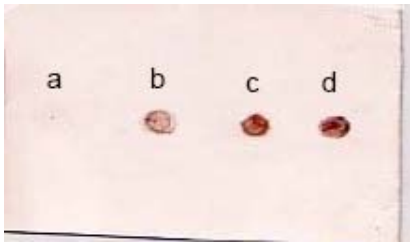

Figure 5. Dot blot assay with antiserum. a: cell extract from cultured *E. Coli* as negative control b: the purified 26 kDa, c: whole cell protein extract, d: intact *H. pylori* cells.

In the present work, a different methodology was used in order to simplify the purification and antibody production against the protein antigen from *H. pylori*. This approach is based on preparative SDS-PAGE, electroelution and antibody production using the natural protein in the gel bands as has been shown in Fig. 2.
Because of the presence of the other thioredoxin dependent peroxiredoxins, purification of AhpC by tracking the enzyme activity was not possible.
While the 26 kDa protein of *H. pylori* has been purified by several multi-step procedures (4, 22-23) the present report provides a simple method of purification. Moreover, several types of apparatus for electroelution are commercially available (25), but this study used only electrophoretic elution using dialysis membrane for protein retention with good results (Table 1 and Fig. 1). On the other hand, this protein band separated on a preparative SDS-PAGE elicited a good immune response in rabbits after immunization with 26 kDa protein in gel, in which polyacrylamide helps to retain the antigen in the injection site and so acts as an adjuvant [26]. In order to titrate the antiserum, indirect ELISA was used in which the purified protein antigen served as a coated antigen. Fig. 3 shows that antigen concentrations lower than 0.2 µg/ml that results in a measurable response can be chosen for coating. For this purpose in this study 0.2 µg of protein antigen per ml was used for this purpose. Fig. 4 shows that the end point titer of antiserum reached to highest point at 1:1000 dilution, while the absorbance values for preimmune serum in the same rabbit is similar to the background. The specificity of the antiserum was further identified by immuno-blotting systems. Fig. 5 shows that in dot blotting assay, in addition to the washed intact bacterial cells, the antiserum reacts with the same purified electroeluted antigen and also with whole cell protein extract of *H. pylori*. The reaction with intact cells showed that a small share of the protein was exposed at the bacterial cell surface. In western blotting system it was found that the antiserum can binds specifically to the dimeric native form of the AhpC (Fig. 6) and indicated that the denatured and native protein shared epitopes. It was also noted that of purified *H. pylori* Tpx of this study did not react with the antiserum (data not shown) which is in agreement with results of another study (27). Taken together, these results suggest that the antibody can be used specifically, to detect the AhpC protein.

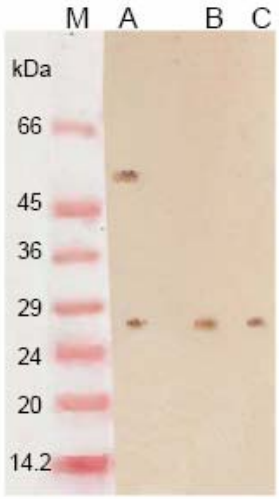

Figure 6. Western blotting with antiserum. Lane M: prestained protein markers, lane A: whole cell protein extract in non- reducing condition, lane B: whole cell protein extract in reducing condition, lane C: the purified 26 kDa protein.

In conclusion, this paper describes a simple method for protein purification and antibody production which avoids both a long term purification of the AhpC protein and addition of Freund's adjuvant. One-dimensional preparative gel electrophoresis allows a single short purification step and the high resolution capacity of this technique leads to a high level of purity of the protein and consequently to a very high specificity of the antibody. Moreover, it avoids contamination by other non-specific proteins which often appear during protein purification by column chromatographic techniques.
To summarize, the present method is simple, rapid and cost-effective and will enables us to produce antibody available for stool antigen EIA in short time and at low cost.

REFERENCES

1. Baker LMS, Raudonikiene A, Hoffman PS, Poole LB. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: Genetics and kinetics characterization. J Bacteriol 2001; 183:1961-1973.
2. Bryk R, Griffin P, Nathan C. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature 2000; 407: 211-215.
3. Wang G, Olczak AA, Walton JP, Maier RJ. Contribution of the *Helicobacter pylori* thiol peroxidase, bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun 2005; 73: 378-384.
4. O' Toole PW, Logan SM, Kostrzynska M, Wadström T, Trust TJ. Isolation and biochemical and molecular analyses of a species-specific protein antigen from the gastric pathogen *Helicobacter pylori*. J Bacteriol 1991; 173: 505-513.
5. Wang G, Conover RC, Benoit S, Olczak AA, Olson JW, Johnson MK, Maier RJ. Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation. J Biol Chem 2004; 279: 51908-51914.
6. Jungblut PR, Bumann D, Haas G, Zimny-Arndt U, Holland P, Lamer S, Siejak F, Aebischer A, Meyer TF. Comparative proteome analysis of *Helicobacter pylori*. Mol Microbiol 2000; 36: 710-725.
7. Chuang M-H, Wu M-S, Lo W-L, Lin J-T, Wong C-H. The antioxidant protein alkylhydroperoxide reductase of *Helicobacter pylori* switches from a peroxide reductase to a molecular chaperone function. Proc Natl Acad Sci USA 2006; 103: 2552-2557.
8. Sheikhian A, Hassan ZM, Mustafaie A, Shokri F, Malekzadeh R, Siavoshi F. Extraction of the outher membrane proteins of *H.pylori* and evaluation of their presence in stool of the infected individuals. Iran Biomed J 2004; 8: 83-88.
9. Mini R, Bernardini G, Salzano AM, Renzone G, Scaloni A, Figura N, Santucci A. Comparative proteomics and immunoproteomics of *Helicobacter pylori* related to different gastric pathologies. J Chromatogr B 2006; 833: 63-79.
10. Chuang M-H., Wu M-S, Lin J-T, Chiou S-H. Proteomic analysis of proteins expressed by *Helicobacter pylori* under oxidative stress. Proteomics 2005; 5: 3895-3901.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature 1970; 227: 680-685.
13. Leppard K, Totty N, Waterfield M, Harlow E, Jenkins J, Crawford L. Purification and partial amino acid sequence analysis of the cellular tumour antigen, p53, from mouse SV40-transformed cells. EMBO J 1983; 2: 1993-1999.
14. Jacobs E, Clad A. Electroelution of fixed and stained membrane proteins from preparative sodium dodecyl sulfate-polyacrylamide gels into membrane trap. Anal Biochem1986; 154: 583-589.
15. Ohhashi T, Moritani C, Andoh H, Satoh S, Ohmori S, Lottspeich F, Ikeda M. Preparative high-yield electroelution of proteins after separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and its application to analysis of amino acid sequences and to raise antibodies. J Chromatogr1991; 585: 153-159.
16. Ball EH. Quantitation of proteins by elution of coomassie brilliant blue R from stained bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 1986; 155, 23-27.
17. Diano M, Le Bivic A, Hirn M. A method for the production of highly specific polyclonal antibodies. Anal Biochem 1987; 166: 224-229.
18. Cumming RH, Iceton G. Cell disintegration and extraction techniques. Chapter 5, In: Protein purification techniques: A practical approach. Roe S ed., 2nd ed., New York: Oxford University Press, 2001; 83-109.
19. Krogfelt KA, Lehours P, Mégraud F. Diagnosis of *Helicobacter pylori* infection. Helicobacter 2005; 10 (suppl.1): 5-13.
20. Dzierżanowska-Fangrat K, Lehours P, Mégraud F, Dzierżanowska D. Diagnosis of *Helicobacter pylori* infection. Helicobacter 2006; 11 (Suppl.1): 6-13.
21. Gisbert JP, Morena F, Abraira V. Accuracy of monoclonal stool antigen test for the diagnosis of *H.pylori* infection: A systematic review and meta-analysis. Am J Gastroenterol 2006; 101: 1921-1930.
22. Lindholm C, Osek J, Svennerholm A-M. Quantification of conserved antigens in *Helicobacter pylori* during different culture conditions. Infect Immun1997; 65: 5376-5380.

23. Papinutto E, Windel HJ, Cendron L, Battistutta R, Kelleher D, Zanotti G. Crystal structure of alkyl hydroperoxide-reductase (AhpC) from *Helicobacter pylori*. Biochem Biophys Acta 2005; 1753: 240-246.
24. Jiang, Z, Tao X-H, Huang A-L, Wang P-L. A study of recombinant protective *H.pylori* antigens. World J Gastroenterol 2002; 8: 308-311.
25. Dunn MJ. Electroelution of proteins from polyacrylamide gels. Chapter 34, In: Protein purification protocols. Cutler P ed., 2nd ed., Totowa, Humana Press Inc, 2004; 339-343.
26. Bugler B, Caizergues-Ferrer M, Bouche G, Bourbon H, Amalric F. Detection and localization of a class of proteins immunologically related to a 100-kDa nucleolar protein. Eur J Biochem 1982; 128: 475-480.
27. Wan X-Y, Zhou Y, Yan Z-Y, Wang H-L, Hou Y-D, Jin D-Y. Scavengase p20: a novel family of bacterial antioxidant enzymes. FEBS letter 1997; 407: 32-36.