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Characteristics of 26 kDa Antigen of *H. Pylori* by Monoclonal Antibody

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ABSTRACT

Alkylhydroperoxide reductase (AhpC, the 26 kDa antigen) is one of the abundant antioxidant enzymes in *Helicobacter pylori* and seems to have a good potential for use in development of immunoassays to detect *H. pylori* infection in clinical specimens. This study aimed to investigate some properties of this antigen by the produced monoclonal antibodies.

Five established hybridoma cell lines secreting monoclonal antibodies (MAbs) against 26 kDa antigen of *H. pylori* were cultivated and MAbs were purified by affinity chromatography. Subsequently, MAbs were conjugated with biotin, and different combinations of capture and tracer antibodies used in sandwich ELISA. Immunoblotting of bacterial extracts were performed to estimate aggregation status of the antigen. Release of antigen from the cultivated bacteria on solid media was examined by sandwich ELISA, and also, existence of interference in fecal extract was investigated by immunoblotting and sandwich ELISA.

Our findings showed that the MAbs against 26 kDa antigen of *H. pylori* could recognize three bands of nearly 25 kDa, 50 kDa, and 75 kDa in immunoblotting. This study also indicated presence of more antigens in the culture medium around the bacteria than the bacterial extract itself. The results of sandwich ELISA and immunoblotting on fecal extracts suggest the presence of interfering agents that prevent detection of antigen by antibody in ELISA but not in immunoblotting.

In this study the oligomerization of the 26 kDa antigen, presence of interfering agents in stool matrix, and release of antigen to outside of bacteria, were demonstrated.

Keywords: Alkyl hydroperoxide reductase; ELISA; *H. pylori*; Immunoblotting; Monoclonal antibody

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INTRODUCTION

Helicobacter pylori is a gram-negative, micro-aerophilic, spiral shaped bacterium that infects about

two-thirds of the world wide population.^{1, 2} HP1563 gene in the *H. pylori* genome encodes a 26 kDa protein that shows alkyl hydroperoxide reductase (AhpC) activity.^{3,4} AhpC is one of the important members of 2-Cys peroxiredoxin (Prx) family that is plentifully expressed in *H. pylori*. This enzyme displays dual functionality under different environmental stress situations; at low-molecular-weight (LMW) oligomers and under microaerobic, short-term oxidative-shock conditions the enzyme has peroxide reductase activity, and at high-molecular-weight (HMW) complexes and during long-term oxidative-stress conditions it has a molecular chaperone activity.^{5,6} Huang et al. reported the AhpC of *H. pylori* is an efficient biomarker for monitoring of *H. pylori* infection.⁶ Additionally comparative proteomic and immunoproteomic analysis of different *H. pylori* strains, demonstrated that AhpC has a potential diagnostic and therapeutic value.⁷

In this study we investigated certain characteristics of 26 kDa antigen of *H. pylori* such as aggregation and release of antigen to outside of bacteria by ELISA and immunoblot methods using monoclonal antibodies.

MATERIALS AND METHODS

Production of Monoclonal Antibody (MAb)

Five specific MAbs against 26 kDa protein of *H. pylori* were prepared as described previously.⁸ Briefly, the designated band of the 26 kDa was isolated from the preparative SDS-PAGE gels and it was cut into pieces. The gel pieces containing the appropriate protein were used for immunization and extraction of the protein using electroelution. BALB/c mice were immunized with the homogenized gel containing 26 kDa antigen, and the purified 26 kDa protein was used for screening of hybridoma supernatants in indirect ELISA. Frozen hybridoma cell lines (A-hel26-A, A-hel26-B, A-hel26-C, A-hel26-D, and A-hel26-E) were revived and cultivated in RPMI 1640 media supplemented with 5-10% FBS (Gibco, USA), penicillin (SERVA, Germany)(100 U/ml) and streptomycin (Sigma-Aldrich, Germany) (100 µg/ml) in 25 and 75 cm² flasks. Supernatants of hybridoma cells were checked with sandwich ELISA for antibody secretion. Microtiter plates (Haghighat, Iran) were coated with 100 µl of an appropriate dilution of goat anti-mouse IgG (Razi Biotech, Iran) in PBS and incubated overnight at 37°C. Nonspecific binding sites were blocked with 100 µl of PBS containing 5% skim

milk (MERCK, Germany) (blocking buffer) for 1 h at 37°C and then 100 µl of each hybridoma supernatant were added to each well. The plates were incubated for 1 h at 37°C, washed thrice with distilled water, and further incubated with 100 µl of an appropriate dilution of HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, Germany) for 1 h at 37°C. After five additional washings, 100 µl of substrate solution (0.64 mM tetramethylbenzidine dihydrochloride (Sigma-Aldrich, Germany) and 2.6 mM H₂O₂ in 0.2 M citrate buffer, pH 6.0) was added. The reaction was stopped after 15 min by the addition of 50 µl of 2 M HCl. The absorbance was measured by a microplate reader at 450 nm. MAb containing supernatants of cultured hybridoma cells were collected and used as antibody resource.

Purification of Antibody

Five purified monoclonal antibodies against 26 kDa antigen of *H. pylori* were obtained from the supernatants of hybridoma cells after purification by salting out and passing through a Protein G-sepharose (Sigma-Aldrich, Germany) affinity column. At first precipitation was performed with an equal volume of saturated ammonium sulfate (MERCK, Germany) and dialysis against phosphate buffered saline (PBS) subsequently it was loaded onto the Protein G-sepharose affinity chromatography column. The column was washed with phosphate buffer (20 mM, pH 7) until the overflow liquid produced no color with Bradford solution. The bound antibody was eluted with 0.1 M glycine-HCl (pH 2.7). The eluted antibodies were neutralized with Tris base (1 M, pH 9), dialyzed against PBS, and stored at -20°C. Purification of MAbs confirmed by reducing SDS-PAGE.

Biotinylation of Antibody

Labeling of antibodies by covalent coupling of a biotinyl group is simple and normally does not have any effect on the antibody.⁹ For this purpose, 0.5 mg of pure antibody was dialyzed against sodium bicarbonate, pH 8.5. Fifty microliters of NHS-biotin (Pro Chem, USA) in DMSO (MERCK, Germany) with concentration of 1 mg/ml was added to antibody and incubated in room temperature for 1 h. Then 20 µl of Tris HCl, 1 M pH 7.5 was added to solution mentioned and incubated at 4°C for 1 h. This solution was dialyzed against three changes of 10 mM PBS to remove free biotin.¹⁰ Biotinylation was confirmed

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using ELISA. After coating with desirable dilution of biotinylated antibody and also unlabeled antibody, microtiter plates were blocked with 100 µl of PBS containing 5% skim milk, and then incubated with 100 µl of an appropriate dilution of HRP-conjugated streptavidin (Sigma-Aldrich, Germany). After washing, 100 µl of substrate solution was added. Biotinylation of antibodies was confirmed by differences of optical density (OD) between wells related to biotinylated and unlabeled antibodies.

Bacterial Strain and Culture Condition

Stomach biopsies in dyspeptic patients were used as *H. pylori* strains. Modified selective media containing Columbia Agar Base supplemented with lysed horse blood, yeast extract, fetal calf serum and M2 medium was used as culture media.¹¹

Bacterial cells from culture plates were harvested in phosphate-buffered saline (PBS, 10 mM, pH 7.2). The suspensions were centrifuged, and the supernatants and pellets were separately stored at -20°C until use.

Preparation of Bacterial Extracts

Frozen cell pellets were thawed, resuspended in 0.2 M glycine hydrochloride (pH 2.2; 4 g of cells/100 ml) supplemented with protease inhibitors [1.0 mM PMSF (Sigma-Aldrich, Germany), 4 mM EDTA (MERCK, Germany)], stirred magnetically for 15min at 20°C, and used as antigen resource. The mentioned collected bacterial washing supernatants were used as another source of antigen.

Immunoblotting of Bacterial Extracts

To confirm the presence of *H. pylori* AhpC in the bacterial extracts and estimation of its changing molecular weight after extraction, immunoblotting was performed. Following discontinuous SDS-PAGE with a 10% separating gel and a 5% stacking gel, the protein bands were transferred onto a PVDF membrane (Bio-Rad, USA). The membrane was blocked and incubated with MAbs for 1.5 h at room temperature. After washing and addition of the HRP (Horse Radish Peroxidase) conjugated second antibody, the bands became visible upon treatment of the blots with diaminobenzidine (DAB) (Sigma-Aldrich, Germany) substrate. Alternatively for immunodetection of bands, biotinylated antibody followed by HRP-conjugated streptavidin was also used.

ELISA Development

The Sandwich ELISA measures the quantity of antigen by two layers of antibodies (capture and tracer antibodies). Each of five purified monoclonal antibodies were used as a capture, and biotinylated antibodies as the tracer antibody in 25 various combinations as shown in table 2. Microtiter plates were coated with desirable dilution of capture antibody and blocked with 100 µl of PBS containing 5% skim milk, followed by incubation with 100 µl of bacterial extract and then an appropriate dilution of tracer antibody, HRP-conjugated streptavidin, and substrate solution, were added, sequentially. Finally, absorbance was measured with a microplate reader at 450 nm. For each combination, PBS containing 0.1% BSA was used to determine the non specific binding.

Antibody A-hel26-E "E" and its biotinylated form "e" were selected as the capture and tracer antibodies, respectively. The standard curve for different dilutions of bacterial extract was constructed. Coating was performed by 10 µg/ml "E" and after blocking with skim milk, microtiter plates were incubated with 100 µl of 1, 1/2, 1/4, 1/8 dilutions of bacterial extract (with concentration of 200 µg/ml) as antigen. After washing, 1/400 dilution of "e" was added, then HRP-conjugated streptavidin and substrate solution were added as noted above.

Release of Antigen of *H. pylori* in Solid Culture Media

As mentioned above, PBS was used to wash the bacterial colonies. To investigate the presence of antigen in this solution, two-fold dilution series (1:1 to 1:8) of the collected bacterial washing supernatants were prepared and used as antigen in sandwich ELISA.

Preparation of Fecal Extract

Four stool samples of patients who had been referred to the clinical laboratory for *H. pylori* stool antigen test collected and stored at -20°C until use. The samples were thawed and completely mixed, then 0.3 g from each stool sample was suspended in 1 ml PBS (20 mM, pH 7.2) and the supernatant was collected by centrifugation at 10000 g for 5 min at room temperature.

Assessment of Interference in Fecal Extract

To assess the presence of interference in fecal extract, detection of antigen in this matrix by ELISA

and immunoblotting was performed. In sandwich ELISA after coating and blocking, microtiter plate was incubated with fecal extract, bacterial extract, and a mixture of them, as antigen, and then HRP-conjugated streptavidin and substrate respectively were used as noted above. The antigens mentioned above were mixed with sample buffer and used in immunoblotting.

RESULTS

Purification of Antibody

MAbs were purified by salting out followed by affinity chromatography with sepharose-4B G-protein. After reduction of disulfide bond using β -mercaptoethanol, two bands with molecular weight of 50 kDa and 25 kDa were detected (Figure 1).

Biotinylation of Antibody

Biotinylation of antibodies was checked by ELISA and confirmed by differences between absorbances of biotinylated and unlabeled antibodies. As shown in table 1, antibodies were biotinylated successfully and they were able to bind to the HRP-conjugated streptavidin in comparison to non-biotinylated antibodies.

Immunoblot Analysis of Bacterial Extracts

Our finding indicated that not only the band of ~25 kDa but also two bands of 45 kDa and 70 kDa could be recognized by MAb against the 26 kDa protein of

H. pylori. It should be noted the bands related to use of biotinylated antibody followed by HRP-conjugated streptavidin, were sharper than using of HRP conjugated second antibody as a reagent for immunodetection (Figure 2).

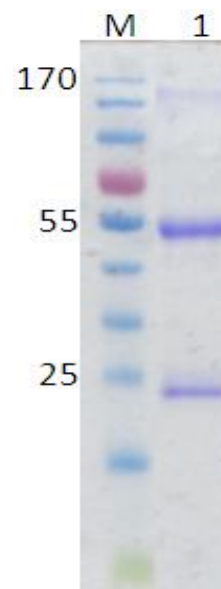


Figure 1. SDS-PAGE analysis of purified MAbs. Lane M, molecular weight markers. Lane 1 represents the A-hel26-E, which was separated under reducing conditions and was stained with Coomassie Brilliant Blue-R250. Bands 50 kDa, and 25k Da represent heavy chain, and light chain of antibody, respectively.

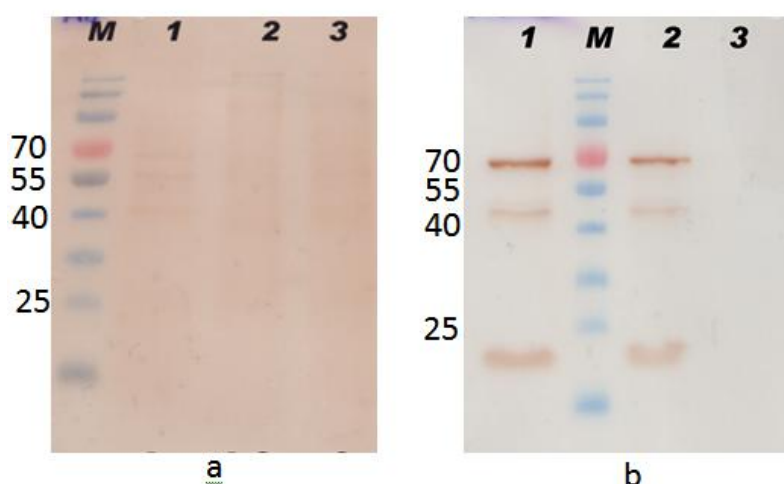


Figure 2. Reaction profiles of MAb A-hel26-E immunoblot analysis with bacterial extracts. Bacterial extract was loaded into the wells. (a) and (b), represent the immunodetection using HRP conjugated second antibody and biotinylated MAb followed by HRP-conjugated streptavidin, respectively. The bands of ~25, 45, and 70 kDa were observed. Lane M, molecular weight markers. Lanes 1, 2, and 3 represent the antigen, antigen-fecal extract, and fecal extract, respectively.

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Table 1. Results of the ELISA for confirmation of antibody biotinylation

MAbs	OD*	
	MAb-B**	MAb***
A-hel26-A	1.83	0.30
A-hel26-B	1.92	0.25
A-hel26-C	1.76	0.22
A-hel26-D	1.85	0.33
A-hel26-E	2.10	0.24

*Optical Density

**Biotinylated Monoclonal Antibody

*** Monoclonal Antibody

To Find the Best Combination of Capture and Labeled Antibodies in Sandwich ELISA

Twenty five combinations of different capture and labeled antibodies were used in sandwich ELISA. The results represent the best combination to be A-hel26-E as capture, and also biotinylated A-hel26-E as labeled antibody (Table 2).

Release of 26 kDa Antigen out of *H. pylori*

Different dilutions of bacterial extract and collected bacterial washing supernatant were used as antigen in sandwich ELISA. The results indicated that the amount of antigen released in washing buffer was more than the antigen in bacterial extract (Table 3).

Table 2. Different combinations of five MAbs against 26 kDa antigen of *H. pylori* as a capture and tracer antibody in sandwich ELISA. Capital letters and Small letters represent capture and tracer antibodies, respectively. The ODs represent net absorbance after subtraction of blank's OD. The best OD is related to E^c combination.

Abs*	OD**	Abs*	OD**	Abs*	OD**	Abs*	OD**	Abs*	OD**
E ^c	1.40	E ^d	0.65	E ^c	1.30	E ^b	0.92	E ^a	0.72
D ^c	0.68	D ^d	0.44	D ^c	0.21	D ^b	0.70	D ^a	0.61
C ^c	0.35	C ^d	0.13	C ^c	0.65	C ^b	0.75	C ^a	0.23
B ^c	0.90	B ^d	0.81	B ^c	0.78	B ^b	0.10	B ^a	0.12
A ^c	1.00	A ^d	0.76	A ^c	0.56	A ^b	0.74	A ^a	0.92

*Antibody Combination

**Optical Density

Table 3. ELISA results for examination of antigen release of *H. pylori* into solid culture media.

Dilution		1/1	1/2	1/4	1/8
OD*	A**	3.3	3.3	2.6	1.6
	B***	2	1.5	1.2	0.9

* Optical Density

** Bacterial Washing Supernatant

*** Bacterial Extract

Detection of Antigen in Fecal Extract by Sandwich ELISA and Immunoblotting

When the distinct amount of 26 kDa antigen was added to the fecal extract, the antigen could not be recognized by the antibodies in sandwich ELISA. Whereas, immunoblotting of mentioned antigens indicated that the antigens were recognized by MAbs (Figure 3).

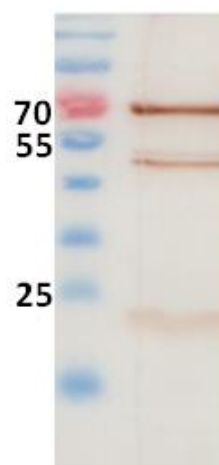


Figure 3. Immunoblotting of fecal extract containing antigen. The same as immunoblotting of antigen in PBS, the bands of ~25, 45, and 70 kDa were observed (Immunodetection was performed by Biotin- Avidin complex).

DISCUSSION

Helicobacter pylori that plays a main role in incidence of peptic ulcers, gastric cancer, and a number of diseases in inner and outer of gastrointestinal tract, infect more than 80% of population of developing countries.¹² AhpC, as one of the abundant antioxidant enzymes in *H. pylori*, possesses dual functionality- peroxidase and chaperone functions- under different environmental stress conditions. *H. pylori* in the presence of this antioxidant enzyme can endure longer in stomach and facilitate carcinogenesis. This study aimed to investigate the some properties of this antigen by the produced MAbs in ELISA and immunoblotting methods. For this purpose, established hybridoma cell lines were cultivated and because the amount of available antigen was very low, antibody secretion of them was examined using sandwich ELISA by two second antibodies against mouse IgG, one of them pure antibody as capture and another one conjugated to HRP as tracer reagents. Purification of MAbs was performed and each of them was conjugated to biotin, then different combinations of five kinds of produced MAbs were checked to choose the best combination of them as capture and tracer antibodies to detect 26 kDa antigen of *H. pylori* in sandwich ELISA method. The best combination was achieved when A-hel26-E was used as capture and also as tracer antibody and it is possible for tandem repeat or oligomeric forms of antigen. This result is consistent with the results reported by Papinutto et al.¹³ about aggregation capability of 26 kDa antigen of *H. pylori*. On the other hand, immunoblotting was performed for estimation of oligomerization status of 26 kDa antigen of *H. pylori*. The results indicated that in addition to the ~25 kDa band, two bands of 45 kDa and 70 kDa were observed, and also immunodetectin by biotinylated MAB followed by HRP-conjugated streptavidin was more specific than using HRP conjugated second antibody. As we mentioned above, in our previous study,⁸ the band of 26 kDa protein of *H. pylori* and also the electroeluted protein of this band, were used for preparation of the MAbs, but in immunoblotting three bands were observed and it is compatible with another reports about oligomerization of AhpC.^{13,14}

Lindholm C et al.¹⁵ examined the quantification of 26 kDa antigen of *H. pylori* in different culture media by inhibition ELISA, while in the present study we investigated release of antigen to out of *H. pylori* in

solid culture media by sandwich ELISA. Our findings represented that a considerable amounts of antigen was released to outside of bacteria.

In fecal extract, the antigen was detected by immunoblotting but was not detected by sandwich ELISA, and these observations suggest the presence of an interfering agent for detectability of antigen in sandwich ELISA. In this way, the given concentration of bacterial extract was added to fecal extract and used in sandwich ELISA and also in immunoblotting. Our results showed that interfering agents prevented detection of antigen in fecal extract in sandwich ELISA but not in immunoblotting. Mucin binding activity of *H. pylori* AhpC was reported by Loke et al.¹⁶ It was also found that the mucin MUC5AC is the primary receptor for *H. pylori*.¹⁷ Thus, our findings suggest that probably the existence of glycoproteins in fecal extract leads to masking of AhpC in ELISA. In immunoblotting, epitops can be detected using antibody because of denaturation of the proteins by SDS and also separation of them in gel.

The oligomerization of the 26 kDa antigen, the presence of interfering agents in stool matrix that prevent detection of antigen by antibody, and release of antigen to outside of bacteria, were shown by immunoblotting and sandwich ELISA.

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