J. Microbiol. Biotechnol. (2000), **10**(1), 56–62

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Expression and Characterization of *Helicobacter pylori* Adhesin Protein Linked to Cholera Toxin A2/B Subunits in *Escherichia coli*

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Received: September 30, 1999

Abstract The *hpa* gene genetically linked to the *ctxa2b* gene was cloned into the pTED expression vector, and the constructed pTED*hpa/ctxa2b* was transformed into *Escherichia coli*. The fusion protein, the adhesin fused to the cholera toxin subunit A2B (CTXA2B) subunit, was expressed to high levels as inclusion bodies in *E. coli*. The expressed protein was partially purified by washing the inclusion bodies with working solution containing 8 M Urea and 0.1 M DTT. Refolding of denatured fusion protein was carried out in the presence of glutathione redox buffer. The refolded fusion protein was purified by size exclusion chromatography. The expressed fusion protein was verified by SDS-PAGE, western blotting with antibodies to both antigenic components of adhesin and cholera toxin subunit B (CTXB), and its N-terminal amino acid sequence was analyzed. The orderly-assembled fusion protein was confirmed by modified G_{M1}-ganglioside ELISA with Abs to adhesin. The results indicate that the purified fusion protein is an Adhesin/CTXA2B protein containing the *H. pylori* adhesin and G_{M1}-ganglioside binding activity of CTXB and the expressed fusion protein in *E. coli* could be easily purified by the refolding process. Its molecular weight was 168 kDa as estimated by size exclusion chromatography. The Adhesin/CTXA2B protein may be used as a candidate antigen for oral immunization against *H. pylori*.

Key words: *Helicobacter pylori* adhesin, Adhesin/CTXA2B, CTXA2B

Helicobacter pylori was first isolated in 1983 by Warren and Marshall [16]. *H. pylori* infection is responsible for chronic active gastritis, peptic ulcers, and gastric cancers [21, 27]. It has been reported that 90% or more of Asians and 60% or more of Europeans are infected with *H. pylori*, although there are local differences [1, 12, 26]. So far, a variety of chemical therapeutic agents such as antibiotics and anti-ulcer agents have been used, in order to treat the gastritis-associated diseases caused by *H. pylori*. However, these drugs have revealed some drawbacks as followings: limitation in penetrating the mucous membrane of stomach, emergence of drug-resistant microorganisms [8], occurrence of reinfection, and side effects of the drugs. Under the circumstances, there are strong reasons for exploring and developing alternative drugs for the control of *H. pylori* by employing new therapeutic approaches, and immunological therapy that can substitute for chemical therapy. An attractive approach to eradicate this infection has been suggested by the therapeutic use of vaccines [9, 27].

Among the components released by *H. pylori*, the adhesin protein may permit the binding of bacteria to gastric cells and their subsequent colonization and, thus, may be important in their pathogenesis. Therefore, the adhesin protein is a potential target protein for therapy and vaccine development against *H. pylori* [5, 6, 11, 13]. The cholera toxin (CTX) is a protein consisting of A and B subunits [19]. The A subunit contributes to intracellular toxicity, whereas the B subunit is required for binding to the eukaryotic cell surface receptor. The A2B subunit of CTX has adjuvant activity, and serves as a carrier protein for orally administered vaccine [7, 24].

In general, *H. pylori* is controlled not by the systemic immune response but by the mucosal immune response, since it colonizes in the gastric mucosa [2, 4, 20, 21]. At present, cholera toxin (CTX) and *E. coli* heat-labile toxin are used as mucosal adjuvants. To stimulate a mucosal immune response, CTX has the most effective adjuvant properties for mucosal immunity. CTX (87 kDa) is composed of toxic A1 subunit (23 kDa), A2 subunit (5 kDa), and five identical subunits of CTXB (11.8 kDa each) that bind to G_{M1}-ganglioside receptors of intestinal cells [3, 18]. CTX is not used in humans because of its toxic effects, but CTXA2B is nontoxic and can be used in humans. Protein antigen is genetically or chemically linked to CTXA2B and elicits secretory (S)-IgA antibodies [10] to provide

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protection at mucosal surfaces by interfering with microbial adherence, colonization, or invasion [28].

In this paper, we report the cloning and sequencing of the *hpa* gene and the fusion gene of *hpa* genetically linked to the *ctxa2b* gene. We also describe the expression, purification, and characterization of the recombinant protein containing the Adhesin/CTXA2B.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli strain XL1-Blue [25] was used as the host for cloning; strain JM101 was used for expression of cloned DNA. For plasmid isolation and recombinant DNA experiments, *E. coli* strains were grown in Luria-Bertani medium with ampicillin (50 μg/ml) at 37°C with vigorous shaking. For expression of cloned DNA, *E. coli* strain JM101 was grown in YCP medium (yeast extract 20g, casamino acid 10 g, KH_2PO_4 3 g, Na_2PO_4 2 g, $(NH_4)_2HPO_4$ 2.5 g, $MgSO_4 \cdot 7H_2O$ 0.24 g, $CaCl_2$ 0.01 g, Glucose 5 g per liter) containing tetracycline (12.5 μg/ml) at 37°C with vigorous shaking. *H. pylori* strain Q-35 is a human clinical isolate obtained from Dr. Kwan-Ho Rhee (Kyung Sang University, Korea). *H. pylori* was passaged on either 8% sheep blood plates (TSA II; BBL) or brucella agar (Difco) plates which were incubated within a BBL GasPak jar containing an anaerobic gas pack (without a catalyst) in a 5% CO_2 incubator [14].

Oligonucleotide and Polymerase Chain Reaction

Oligonucleotide primers were synthesized by using an Applied Biosystems DNA Synthesizer (model 380 A). The primers used to amplify the *hpa* gene were 5'-CCGTGG-CTCAAGCTGCATGGAAAAAATGCCTTTTAGG-3' (underline containing *Dsa*I site) and 5'-AGAATTCTCGGT-TTCTTTTGCCTTTTAATT-3' (underline containing *Eco*RI site). The primers used to amplify the *ctxa2b* gene were 5'-AGAATTCGAAGAGCCGTGGATTCATCAT-3' (underline containing *Eco*RI site) and 5'-ACTGCAGCACATAATA-CGCACTAAGGA-3' (underline containing *Pst*I site). The *hpa* and *ctxa2b* genes were amplified from *H. pylori* and plasmid pRIT10814 (clone 39052 containing the *ctx* gene; American Type Culture Collection, Rockville, U.S.A.) by PCR, respectively. Amplification of the *hpa* and *ctxa2b* genes were performed as previously described, and were verified by digestion of the amplified DNA with several restriction endonuclease, chosen according to the published *hpa* and *ctxa2b* nucleotide sequences [5, 28]

Construction of an *hpa* and *ctxa2b* Fusion Gene Expression Vector

H. pylori chromosomal DNA was isolated as described by Majewski and Goodwin [15]. Amplified *hpa* and *ctxa2b* genes were digested with *Dsa*I and *Eco*RI, and the *hpa* and *ctxa2b* genes were cloned into the pBluescript II SK (+) (Stratagene Ltd., Cambridge, U.K.). The resulting constructs were named as pBluehpa and pBlue*ctxa2b*, respectively. The correct nucleotide sequence and in-frame sequence were verified by nucleic acid sequencing (Sequenase version 2.0 DNA sequencing kit; USB™). The amino acid sequences of the *hpa* and *ctxa2b* genes were deduced. The pBlue*ctxa2b* was digested with *Eco*RI and *Pst*I, and the 701 bp DNA fragment generated by the restriction endonucleases was ligated into the pBlue*hpa* vector and the resulting construct was named pBlue*hpa/ctxa2b*. The *hpa/ctxa2b* gene fragment generated by digesting pBlue*hpa/ctxa2b* with *Dsa*I and *Pst*I was cloned into an expression vector, pTED [22], and the resulting construct was named pTED*hpa/ctxa2b*. The plasmid pTED*hpa/ctxa2b* was used to transform *E. coli* JM101. Transformation was carried out by electroporation according to the manufacturer's directions (Gibco BRL Life Technologies, Inc., Gaithersburg, U.S.A.) [25].

Synthesis of the Peptide for the Receptor-Binding Motif of Adhesin and Production of Antiserum

The peptide corresponding to the putative sialic acid binding motif of adhesin (Leu-Arg-Pro-Asp-Pro-Lys-Arg-Thr-Ile-Gln-Lys-Lys) was synthesized by Multiple Peptide Systems (TAKARA SHUZO Co., Ltd. Japan). Rabbits were injected with 100 μg of protein carrier (keyhole limpet hemocyanin)-conjugated peptide in Freund's complete adjuvant. After receiving two booster injections of 100 μg of synthetic peptide, the antibody was isolated. A protocol similar to that described by Evans and coworkers was applied [5].

Expression and Purification of Fusion Protein

Growth of recombinant *E. coli* JM101 carrying pTEDhpa/*ctxa2b* was conducted in YCP media containing 12.5 μg/ml tetracycline at 37°C. Target gene expression was induced at midlog phase by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). The cells were grown for 4 h after IPTG induction and harvested by centrifugation. The cells were resuspended in 3 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl)/g wet weight of bacterial pellet. PMSF (1 mM) and lysozyme (0.4 mg/ml) were added to the cell suspension after 40 min of incubation at 4°C, and the suspension was treated with sodium deoxycholate (1.33 mg/ml) at room temperature until it became viscous. RNase A and DNase I were added to a final concentration of 10 μg/ml and $MgCl_2$ to 5 mM. After a 40-min incubation at room temperature, the mixture was centrifuged at 14,000 ×g for 20 min at 4°C. The resulting pellet, which contained insoluble recombinant fusion protein as inclusion bodies, was washed with buffer (50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 1 mM EDTA,

0.5% Triton X-100). After centrifugation at 14,000 ×g for 20 min, washing buffer containing 8 M urea and 0.1 M DTT was added and the suspension was incubated for 60 min at room temperature to solubilize the pellet. The supernatant was recovered by centrifugation at 14,000 ×g for 20 min at 4°C. This denatured fusion protein renatured by dialysis against dilute working solution containing a glutathione redox buffer (5 mM GSH and 0.5 mM GSSH) for 16 h at 4°C. The supernatant containing renatured fusion protein was subjected to size-exclusion chromatography on an FPLC TSK-GEL G-3000SW column (21.5×300 mm; TosoHaas, Montgomeryville, U.S.A.), equilibrated with working solution. The protein yield was estimated by the Bradford assay method using BSA as the standard [19, 23].

SDS-PAGE and Western Blotting Analysis of Fusion Protein

SDS-PAGE (pre-cast 10-20% gradient Tris-Tricine gel, Novex) and western blotting were used to examine the purified fusion protein [25]. Proteins were resolved by electrophoresis and stained with Coomassie blue. For western blots, a gel run in parallel was electrotransferred onto PVDF membrane. The blots were probed with antibody to CTXB (List Biologic Laboratories, Cambell, U.S.A.) and peptide containing the receptor-binding motif of adhesin. Proteins on the blot were detected using alkaline phosphatase-conjugated goat antiserum to mouse IgG (Caltag Laboratories Inc., South San Francisco, U.S.A.), and developed using a BCIP/NBT (Sigma) substrate system (Kirkegard and Perry Labs, Gaithersburg, U.S.A.).

Modified G_{M1}-Ganglioside ELISA

To determine properly assembled fusion protein, binding to G_{M1}-ganglioside (from Bovine brain) in ELISA plate was performed [7, 24]. Wells were coated overnight at room temperature with 100 μl/well of 1.5 μM G_{M1}-ganglioside in PBS and then washed with PBS. Purified fusion protein was diluted with an assay buffer (PBS containing 0.05% Tween-20 and 0.5% BSA) and transferred to the coated wells of the plates. The plates were incubated at 37°C for 120 min. After the wells were washed three times, the plates were incubated with rabbit antibody to peptide containing the receptor-binding motif of adhesin at 37°C for 120 min and then with alkaline phosphatase-conjugated goat antiserum to rabbit IgG. After incubation for 120 min at 37°C, the plates were developed with pNPP (para-nitrophenyl phosphate) solution and the absorbance was read at 405 nm in an ELISA reader.

N-Terminal Amino Acid Sequence

The purified fusion proteins were resolved by electrophoresis in SDS-PAGE gels. The protein bands corresponding to Adhesin/CTXA2 and CTXB were transferred to a PVDF membrane and subjected to NH_2-terminal sequencing (model Procise 491 Sequencer, Applied Biosystems, Inc., Foster City, U.S.A.).

Results and Discussion

Cloning and Expression of the *hpa/ctxa2b* Gene

The *hpa* gene was amplified by PCR using *H. pylori* chromosomal DNA as template. The amplified *hpa* gene fragment, containing *Dsa*I and *Eco*RI restriction sites, was cloned into pBluescript II SK (+) yielding pBluehpa. The nucleotide sequences of both strands were determined for the 771 bp *Dsa*I/*Eco*RI *hpa* fragment of pBluehpa. The nucleotides homology search indicated 94.3% identity with the *hpa* gene. Majewski and coworker reported that the *H. pylori* genome was the variable gene according to strains [15]. The amino acid sequence KRTIQK (amino acid residues 124 to 129) corresponded to the sialic acid binding sequence described by Evans and coworkers [5]. The *ctxa2b* gene was amplified by PCR using plasmid pRIT10814 as template and the PCR products, containing *Eco*RI and *Pst*I restriction sites, were cloned into pBluescript II SK (+) yielding pBlue*ctxa2b*. The pBlue*ctxa2b* was excised with *Eco*RI and *Pst*I, and the 701 bp DNA fragment was ligated into the pBlue*hpa* vector and named as pBlue*hpa/ctxa2b*. The *hpa/ctxa2b* gene (1,480 bp) fragment generated by digesting pBlue*hpa/ctxa2b* with *Dsa*I and *Pst*I was cloned into the expression vector, pTED, and named pTED*hpa/ctxa2b*. The structure of the plasmid encoding the fusion protein is shown in Fig. 1.

The protein produced by recombinant *E. coli* JM101 carrying pTED*hpa/ctxa2b* was examined by SDS-PAGE (Fig. 2A). SDS dissociated the protein into OmpA/Adhesin/CTXA2B and CTXB, which disrupts its pentameric structure. No other protein bands were detected using the highly sensitive silver staining, which indicated its high purity. The co-expressed OmpA/Adhesin/CTXA2 and CTXB were specifically detected by Western blot analysis using antibody to peptide containing the receptor-binding motif of adhesin and to CTXB (Fig. 2B). It was expected that the pTED*hpa/ctxa2b* construct would be transcribed as one message and translated as two proteins, as in the case with the native *ctx* operon [19]. A fusion protein consisting of the vector-derived ompA signal peptide, the adhesin, and the CTXA2 peptide would be expressed by means of the vector translation initiation signals and a stop codon at the 3' end of the CTXA2 coding sequence.

Isolation and Denaturation of Inclusion Bodies Containing Fusion Protein

Recombinant *E. coli* were grown, harvested, and disrupted. A protein with G_{M1}-ganglioside-binding activity and adhesin antigenity was detected in the cytoplasmic extract. Because this cytoplasmic extract also contained other cellular

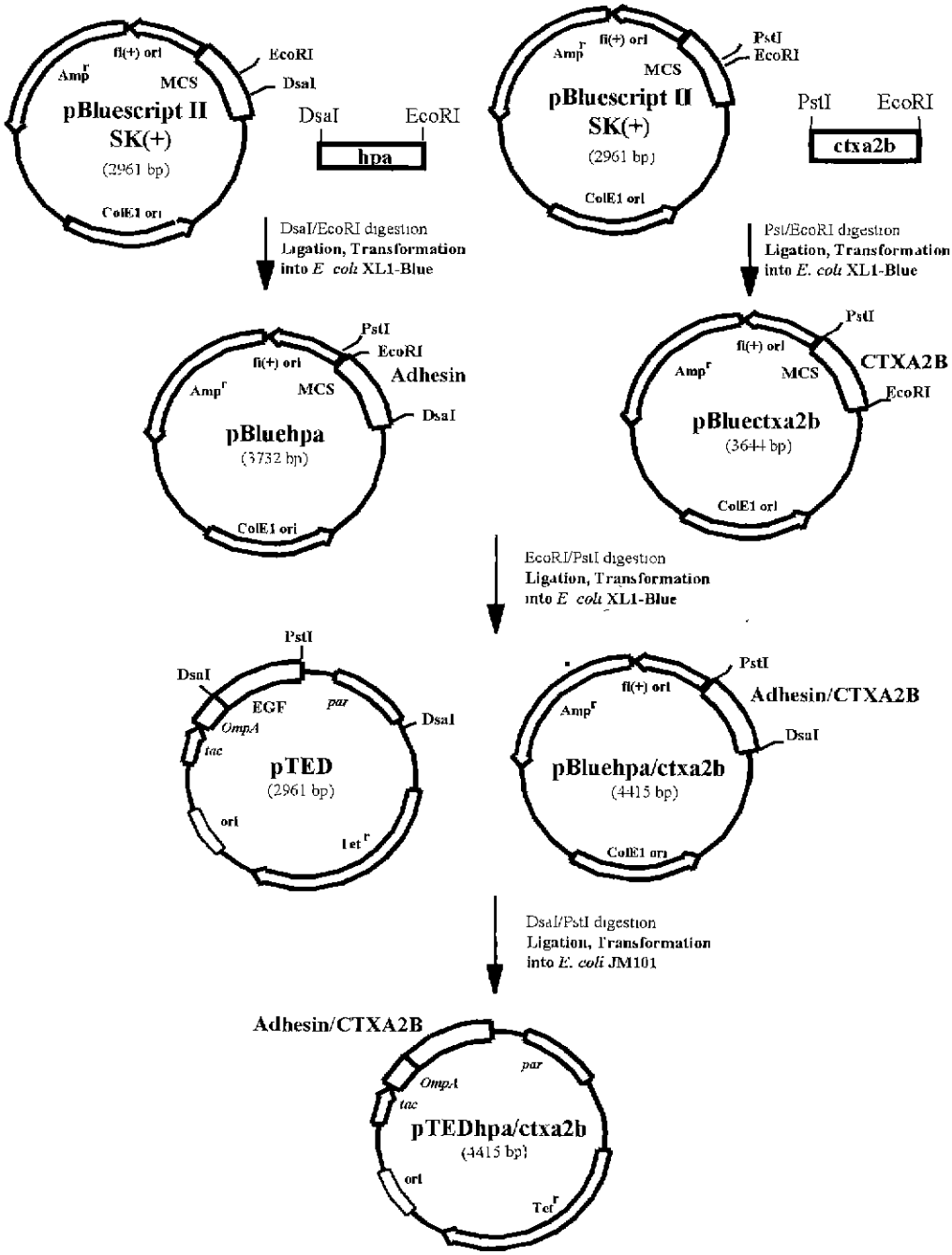

Fig. 1. Scheme of construction of the pTED*hpa/ctxa2b* expression vector.
The pBlue*hpa* vector was constructed by the PCR products of the *hpa* gene from *H. pylori* chromosomal DNA into the pBluescript II SK(+). The pBlue*ctxa2b* vector was constructed by the PCR products of the *ctxa2b* gene from pRT10814 into the pBluescript II SK(+). The pBlue*hpa/ctxa2b* vector was constructed by the pBluehpa and *ctxa2b* gene from pBluectxa2b The *hpa/ctxa2b* gene was excised from the pBlue*hpa/ctxa2b* with *DsaI* and *PstI*, and cloned into the *DsaI* and *PstI* sites of pTED vector Abbreviations ColE1 *ori*, origin of replication; *tac*, tac promoter; *OmpA*, signal peptides of secretion; *Amp*, ampicillin; *Tet*, tetracycline.

components (Fig. 3, lane 2), the fusion proteins were further purified by a series of steps using 0.5% Triton X-100. But these solubilizing conditions did not appear to solubilize the fusion protein (Fig. 3, lanes 2-7). The partially purified, insoluble pellets were not solubilized in 2 M urea (Fig. 3, lane 9), but solubilized in 8 M urea or 8 M urea/0.1M DTT (Fig. 3, lanes 10-11). The fusion protein was detected in the cytoplasmic extract as inclusion bodies, although the chimeric proteins, the OmpA signal peptide, existed in the expression vector. In *V. cholerae,*

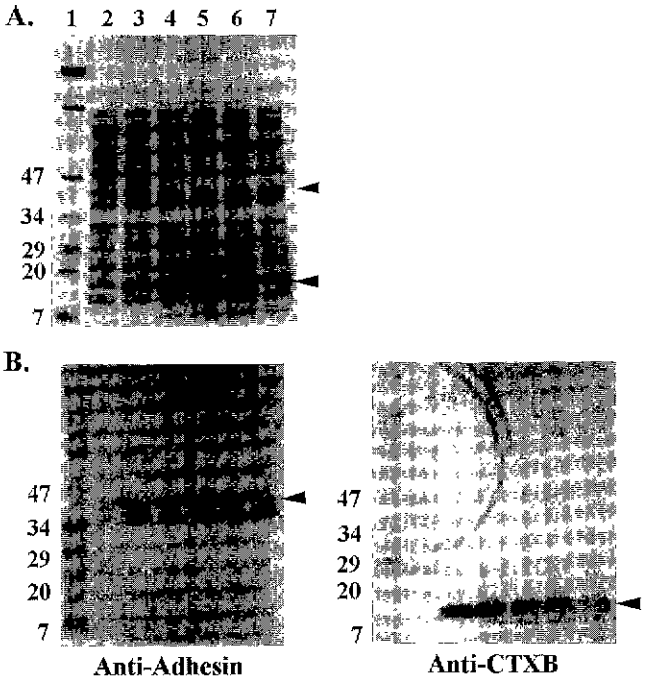

Fig. 2. SDS-PAGE of total proteins from *E. coli* JM101 carrying pTED*hpa/ctxa2b* (A), and western blots with polyclonal antibody to peptide containing the receptor-binding motif of adhesin and to CTXB (B).

Lane 1, prestained molecular weight marker. Cell lysate proteins (lanes 2-7) from *E. coli* JM101 carrying pTED*hpa/ctxa2b* after 0, 1, 2, 4, 6, 24 h induction with 1 mM IPTG were resolved by electrophoresis on a pre-cast 10-20% gradient polyacrylamide gel and immunoblotted on a PVDF membrane. The PVDF membrane was exposed to 1:1,000 dilutions of polyclonal antibody to a sequence of amino acids including the receptor-binding motif of adhesin or 1:2,000 dilutions of a polyclonal antibody to CTXB. In all cases, samples applied to the gel were equivalent to 5 µl of cell lysates. Arrowhead indicates Adhesin/CTXA2 and CTXB.

native CTXB was synthesized as a cytoplasmic precursor polypeptide that is exported across the inner membrane and processed by removal of the signal sequence to mature protein which, subsequently, will assemble spontaneously into pentamers in the periplasm [17]. It has been reported that *V. cholerae,* but not *E. coli*, could secrete the native CTXB into the culture medium by translocating these protein complexes across the outer membrane [23].

Refolding and Purification of the Fusion Protein

Because the cytoplasm of bacteria such as *E. coli* is maintained in a reduced state [17], and oxidation of CTX subunits is required for assembly [23], it is possible that cytoplasmic Adhesin/CTXA2B was formed *in vitro*. The fusion proteins denatured by 8 M urea or 8 M urea and 0.1 M DTT were refolded by diluting the 8 M urea to a final concentration of 0.2 M urea containing a glutathione redox buffer (5 mM GSH and 0.5 mM GSSH). The fusion proteins denatured by 8 M urea did not refold, unless the 8 M urea contained 0.1 M DTT (data not shown).

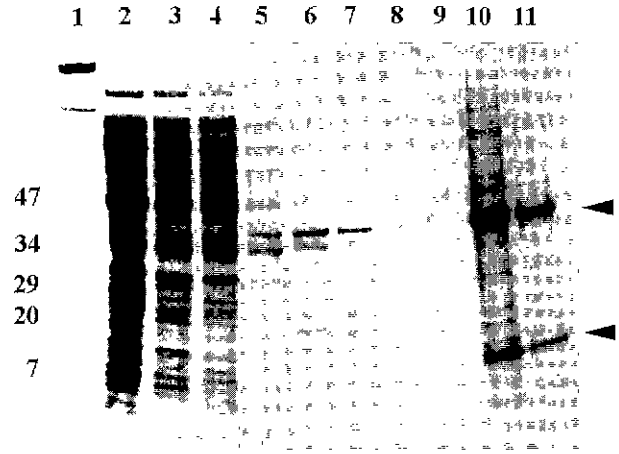

Fig. 3. SDS-PAGE monitoring of the isolation of inclusion bodies in *E. coli* JM101 carrying pTED*hpa/ctxa2b*.

SDS-PAGE was performed on a pre-cast 10-20% gradient polyacrylamide gel and stained with Coomassie brilliant blue R250. Lane 1, standard protein marker; lane 2, whole cell lysates; lane 3, first washing with 0.5% Triton X-100 in working solution; lane 4, second washing with 0.5% Triton X-100 in working solution; lane 5, third washing with 0.5% Triton X-100 in working solution, lane 6, fourth washing with 0.5% Triton X-100 in working solution, lane 7, fifth washing with 0.5% Triton X-100 in working solution lane 8, sixth-washing with 0.5% Triton X-100 in working solution; lane 9, washing with 2 M Urea in working solution, lane 10, washing with 8 M Urea in working solution; lane 11, washing with 1 mM DTT and 8 M Urea in working solution. Arrowhead indicates Adhesin/CTXA2 and CTXB.

Refolded fusion proteins were purified by size-exclusion chromatography on an FPLC TSK-GEL G-3000 SW column. Correct assembly of Adhesin/CTXA2B was monitored by G_{M1}-ganglioside ELISA developed with antibody to peptide containing the receptor-binding motif of adhesin (Fig. 4A). SDS-PAGE and Western blots were used to analyze the purified fractions (Fig. 4B). The purified Adhesin/CTXA2B was estimated to be 92% pure by densitometric assay. The molecular weight of Adhesin/CTXA2B predicted by the amino acid sequence was 96 kDa (OmpA/Adhesin/CTXA2B). To estimate the molecular weight, purified fusion protein was subjected to size-exclusion chromatography on an HPLC column calibrated with molecular standard markers. The eluted proteins indicated a 168 kDa molecular weight which was about twice the expected value. Whether the fusion proteins can associate to create a dimeric molecule or whether the molecular shape of adhesin is responsible for the anomalous elution is not known.

Characterization of the Fusion Protein

The purified fusion proteins were confirmed by N-terminal amino acid sequence analysis of Adhesin/CTXA2 and CTXB bands on the SDS-PAGE (data not shown). The N-terminal amino acid sequences of adhesin protein and CTXB were Met-Lys-Lys-Thr-Ala-Ile-Pro-Ser-Val-Gln and Met-Ile-Lys-Leu-Lys-Phe-Gly-Val-Glu-Phe, respectively.

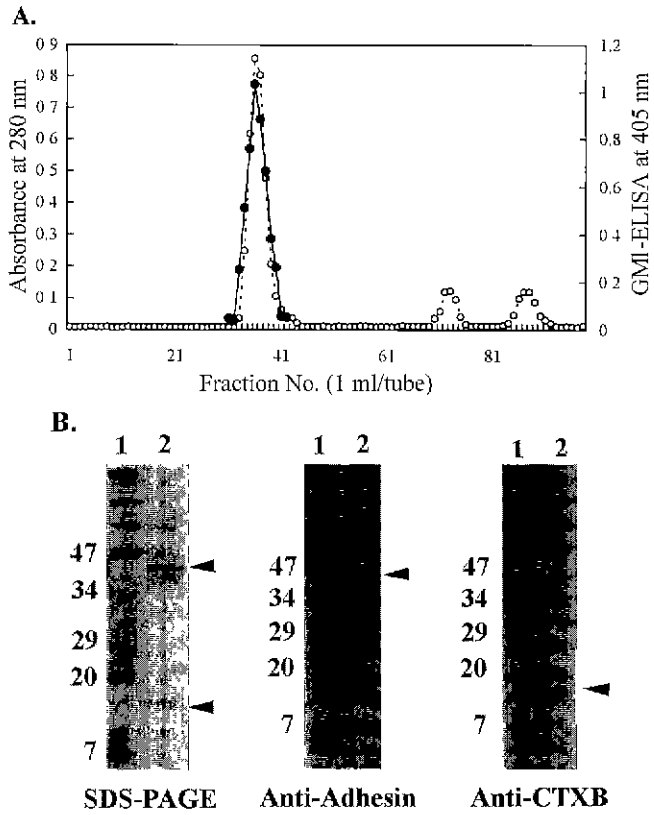

Fig. 4. Size exclusion chromatography of refolded Adhesin/CTXA2B proteins on TSK column (A). SDS-PAGE and Western blots were used to analyze the purified fractions (B). The column (TSK-GEL G-3000 SW column; 21.5×300 mm Toso-Haas Co.) was equilibrated with working solution (50 mM NaH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 8.5). The flow rate was 1 ml/min. The ○ stands for the absorbance at 280 nm. The ● stands for the absorbance at 405 nm.

These sequences were signal peptides of OmpA/Adhesin/CTXA2 and CTXB. To determine properly assembled fusion protein, the purified Adhesin/CTXA2B molecule was tested in modified G_{M1}-ganglioside ELISA developed with polyclonal antibodies to peptide containing the receptor-binding motif of adhesin. As shown in Fig. 5, the purified Adhesin/CTXA2B was able to bind G_{M1}-ganglioside, suggessting that it possesses adhesin epitopes. The data also indirectly showed that the CTX bound to G_{M1}-ganglioside no longer possessed adhesin epitopes and did not have the affinity for the CTXB binding site. There is evidence to suggest that conjugation of antigens to CTXB improves the immunogenicity of such preparations when they are administered orally [18]. On the basis of the previous data, the Adhesin/CTXA2B protein may be used as a candidate antigen for oral immunization against *H. pylori*.

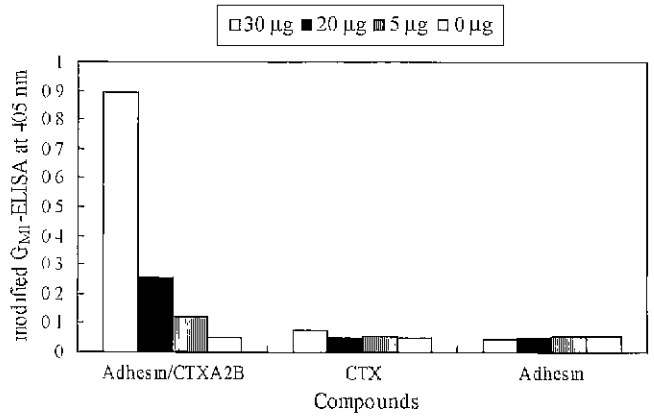

Fig. 5. Comparison of the affinity of the Adhesin/CTXA2B, CTX, and Adhesin for G_{M1}-ganglioside by competitive ELISA.
A modified G_{M1}-ganglioside ELISA was performed as described in Materials and Methods. This experiment was repeated three times with similar results.

Acknowledgments

The authors would like to thank Dr. Eugene P. Mayer for helpful discussions and for critically reviewing the manuscript. This paper was supported by the faculty research funds of Sungkyunkwan University, 1998.

References

1. Blaser, M. J. 1990. Epidemiology and pathophysiology of *Campylobacter pylori* infections. *Rev. Infect. Dis.* **12:** S99-S106.
2. Bruno, G., M. Catherine, F. Sophine, H. Jean, V. L. Elisabeth, and R. Bachre. 1998. Systemic immunization with urease protects mice against *Helicobacter pylori* infection. *Vaccine* **16:** 850-856.
3. Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren. 1989. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. *Infect. Immun.* **57:** 1072-1081
4. Czinn, S. J., A. Cai, and J. G. Nedrud. 1993. Protection of germ-free mice from infection by *Helicobacter felis* after active oral or passive IgA immunization. *Vaccine* **11:** 637-642.
5. Evans D. G., T K. Karjalainen, D. G. Evans Jr., D. Y. Graham, and C. H. Lee. 1993. Cloning, nucleotide sequence, and expression of a gene encoding an adhesin subunit protein of *Helicobacter pylori*. *J. Bacteriol.* **175:** 674-683.
6. Evans, D. J., D. G. Evans, K. E. Smith, and D. Y. Graham. 1989. Serum antibody responses to the N-acetylneuraminyllactose-binding of *Campylobacter pylori*. *Infect Immun* **57:** 664-667.
7. Hajishengallis, G., S. K. Hollingshead, T. Koga, and M. W. Russell. 1995. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. *J. Immunology* **154:** 4322-4332.
8. Glupczynski, Y., A. Burette, H. Goossens, C. DePrez, and J. P. Butzler. 1992. Effect of antimicrobial therapy on the

specific serological response to *Helicobacter pylori* infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **11:** 583–588.
9. Glupczynski, Y. and A. Burette. 1992. Eradicating *Helicobacter pylori*. *Lancet* **339:** 54–55.
10. Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. *Nature* **292:** 413–417.
11. Jones, A., C. J. Luke, C. Cockayne, D. J. Constantinidou, and C. W. Penn. 1995. Gene cloning of a flagellar sheath protein of *Helicobacter pylori*, p. 64. *In* Newell, D. G., J. Ketley, and R. A. Feldman (eds.). *8th International Workshop on Campylobacter, Helicobacter and Related Organisms*. Central Veterinary Laboratory, Winchester, United Kingdom, U.K.
12. Lee, K. K., J. S. Son., Y. J. Chang., S. U. Kim, and K. H. Kim. 1998. Separate expression and *in vitro* activation of recombinant *Helicobacter pylori* urease structural subunits. *J. Microbiol. Biotechnol.* **8:** 700–704.
13. Lingwood, C. A., G. Wasfy, H. Han, and M. Huesca. 1993. Receptors affinity purification of a lipid-binding adhesion from *Helicobacter pylori*. *Infect. Immun.* **61:** 2474–2478.
14. Lingwood, C. A. 1993. *Helicobacter pylori*: Receptors and adhesins, pp. 209–222. *In* Goodwin, C. S. and B. W. Worsley (eds.), *Helicobacter pylori*: *Biology and Clinical Practice*. CRC Press Inc , Boca Raton, FL, U S.A.
15. Majewski, S. I. H. and C. S. Goodwin. 1988. Restriction endonuclease analysis of the genome of *Camphylobacter pylori* with a rapid extraction method: Evidence for considerable genomic variation. *J. Infect. Dis.* **157:** 465–471.
16. Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren, and J. A. Armstron. 1984. Original isolation of *Campylobacter pylordis* from human gastric mucosa. *Microbios. Lett.* **25:** 83–88.
17. Marston, F. A. O. 1986. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240:** 1–8.
18. McKenzie, S. J. and J. F. Halsey. 1984. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J. Immunol.* **133:** 1818–1823.
19. Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde. 1983. Cholera toxin genes: Nucleotide sequence, deletion analysis and vaccine development. *Nature* **306:** 551–559.
20. Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune response in external secretions. *J. Clin. Immunol.* **7:** 265–272.
21. Michetti, M., M. Rossi, V. Giannelli, M. M. Giuliani, M. Pizza, S. Censini, A. Covacci, P. Massari, C. Pagliaccia, R. Manetti, J. L. Telford, G. Douce, G. Dougan, R. Rappuoli, and P. Ghiara. 1998. Protection against *Helicobacter pylori* infection in mice by intragastric vaccination with *H. pylori* antigens is achieved using a non-toxic mutant of *E. coli* heat-labile enterotoxin (LT) as adjuvant. *Vaccine* **16:** 33–37.
22. Park, M. H., S. K. Park, Y. H. Yu, Y. W. Koh, C. H. Kown, S. H. Kim, J. Y. Jung, S. W. Yoon, and T. Y. Koo. 1995. Large-scale production of recombinant human epidermal growth factor and its development as a new drug, pp. 63–72. *Proceedings of International Symposium on R&D in the Pharmaceutical Industry of Korea and Hungary*, Budapest, Hungary.
23. Philippe, S. D., S. N. Accart, H. V. J. Kolbe, D. Schubnel, B. Bernadette, R. Bischoff, and K. Marie-Paule. 1994. Recombinant cholera toxin B subunit in *Escherichia coli*: High-level secretion, purification, and characterization. *Protein Expres. Purif.* **5:** 518–526.
24. Russell, M. W. and H. Y. Wu. 1991. Distribution, persistence, and recall of serum and salivary antibody responses to peroral immunization with protein antigen I/II of *Streptococcus mutans* coupled to the cholera toxin B subunit. *Infect. Immun.* **59:** 4061–4074.
25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
26. Taylor, D. N. and M. J. Blaser. 1991. The epidemiology of *Helicobacter pylori* infection. *Epidemiol. Rev.* **13:** 42–59
27. Telford, J. L., A. Covacci, P. Ghiara, C. Montecucco, and R. Rappuoli. 1994. Unravelling the pathogenic role of *Helicobacter pylori* in peptic ulcer; potential for new therapies and vaccines. *Trends Biotechnol.* **12:** 420–426.
28. Wu, H. Y. and M. W. Russell. 1993. Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. *Infect. Immun* **61:** 314–320.