

Production of anti-*Helicobacter pylori* urease-specific immunoglobulin in egg yolk using an antigenic epitope of *H. pylori* urease

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The potential therapeutic effects of *Helicobacter pylori*-specific immunoglobulin (IgY-*Hp*) derived from egg yolk and identification of the immunodominant *H. pylori* proteins have previously been reported. In this study, the urease epitope that is recognized by IgY-*Hp* was identified and used as an immunogen to produce urease-specific IgY (IgY-*HpU*). Epitope regions were mapped and peptides of selected epitope regions were synthesized. The IgY-*Hp* titre against synthetic peptides was evaluated using ELISA analysis. Hens were immunized with synthetic peptides conjugated with BSA. Urease activity was quantified by measuring the optical density of an indicator dye. Of the five synthetic peptides assayed, a peptide representing 15 amino acid residues of UreB (UB-3; aa 396–410, DNDNFRIKRYLSKYT) was specifically recognized by the IgY-*Hp*. Immunization of hens with BSA-conjugated UB-3 resulted in the generation of IgY-*HpU*. IgY-*HpU* markedly reduced *H. pylori* urease activity by 80 % as compared to control IgY (IgY-BSA). The availability of the synthetic UreB-derived peptide enabled the production of highly specific anti-urease IgY, which had a significant inhibitory effect on *H. pylori* urease activity. Therefore, specific IgY-*HpU* produced using the synthetic peptide may be an effective tool against infection by *H. pylori*.

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INTRODUCTION

Current triple therapies are effective against *Helicobacter pylori* infection, but the development of antibiotic resistance and the challenge of re-treatment after initial failure demand that novel approaches be developed (Deloney & Schiller, 2000; Peitz *et al.*, 1998). Vaccines (Kusters, 2001; Del *et al.*, 2001), probiotics (Aiba *et al.*, 1998), natural extracts (Mabe *et al.*, 1999; O’Gara *et al.*, 2000), anti-adhesion compounds and mucosal protective agents (Mysore *et al.*, 1999) are under investigation. Development of a vaccine is the basic approach to *H. pylori* treatment, but efficacy data are still lacking in humans (Kusters, 2001). *H. pylori* is able to bind to several receptors at the surface of gastric epithelial cells (Guruge *et al.*, 1998; Taylor *et al.*, 1998); thus prevention of *H. pylori* binding to gastric epithelial cells could represent a potential target for *H. pylori* treatment. We previously reported that egg-yolk-derived immunoglobulin (IgY) obtained from hens that were immunized with *H. pylori* whole-cell lysate may provide a novel alternative approach to the treatment of *H. pylori* infection (Shin *et al.*, 2002). However, many problems remain with regard to clinical applications, such as the cross-reactivity to normal flora and the mass-production of vaccine. IgY produced using whole-cell lysates presents the likelihood of cross-reactivity with other bacteria, including the normal human flora (Shin *et al.*, 2003), which could decrease the efficacy of IgY-*Hp*. Thus, immunization using a

selective antigen is required. Also, to mass-produce IgY-*Hp* using a selective antigen as the immunogen, mass culturing of *H. pylori* or recombinant DNA techniques are needed, but these processes are time-consuming and labour-intensive. In a previous study, we elucidated the five immunodominant *H. pylori* proteins that react strongly with IgY-*Hp* to address the limitations of IgY-*Hp* (Shin *et al.*, 2003). Among the immunodominant proteins identified was urease, a well-known critical virulence factor (Bury-Mone *et al.*, 2001) and the antigen most often studied in vaccine trials.

In this study, to produce specific anti-urease IgY (IgY-*HpU*), we identified the epitope of *H. pylori* using several synthetic peptide fragments that were predicted as possible epitopes. The synthetic peptide identified as the epitope was used as the immunogen for IgY-*HpU* production. In addition, the ability of the IgY-*HpU* to inhibit urease activity was evaluated.

METHODS

Epitope mapping and peptide synthesis. The urease epitope that was recognized by IgY-*Hp*, previously obtained from hens by immunization with *H. pylori* whole-cell lysates, was predicted using protein hydropathy plots constructed according to the Kyte–Doolittle scale. Domains with a high degree of hydrophilicity were selected as peptide antigens. Two 15-residue peptides selected from amino acids 1–238 of

UreA (UA-1, residues 18–32 and UA-2, residues 216–230) and three peptides of 15–16 residues selected from amino acids 1–569 of UreB (UB-1, residues 52–67; UB-2, residues 381–395; and UB-3, residues 396–410) were synthesized by the solid phase method of Merrifield (1965). In brief, the tert-butyloxycarbonyl-protected amino acids were obtained using a multipetide synthesizer (Applied Biosystems). Each peptide was partially unblocked and cleaved from the resin. All synthetic peptides were purified and isolated by reverse-phase (RP-HPLC) performed with a liquid chromatograph (Thermo Separation Products). Peptide mass was analysed by RP-HPLC (LC/MSD).

Peptide ELISA (pELISA). To assess the reactivity of IgY-*Hp* to the synthetic peptides, we performed ELISA as described by Akita & Nakai (1992) with modifications. Reacti-Bind 96-well polystyrene plates activated with maleic anhydride (Pierce Biotechnology) were used to immobilize the synthesized peptides. The 96-well plates were coated overnight at 4 °C with each peptide solubilized in 0.1 M carbonate buffer (pH 9.6) at a concentration of 10 µg per well. After blocking with 1% (w/v) BSA, 100 µl of a 1 : 1000 dilution of IgY-*Hp* (1 mg ml⁻¹) was added. The plates were then washed with PBS, pH 7.2, containing 0.05% (v/v) Tween 20 (PBS-Tween). Alkaline phosphatase-conjugated goat anti-chicken IgY (Promega) was added, and the plates were incubated for 1 h. The plates were washed with PBS-Tween, and disodium *p*-nitrophenyl phosphate (Sigma) was added to each well as a substrate. After incubation for 10 min, the reaction was stopped by the addition of 3 M NaOH. The A₄₀₅ was measured using a microplate reader (Labsystems Multiskan MS).

Immunization. Brown Leghorn hens (25 weeks old; *n* = 15) were immunized intramuscularly with the BSA-conjugated peptides (500 µg ml⁻¹) using an equal volume of Freund's complete adjuvant (Difco). The leg muscle of each hen was injected at four different sites (250 µl per site). Three booster injections, with Freund's incomplete adjuvant, were given at 2-week intervals following the first injection. One month after immunization, the eggs were collected daily for 1 month and stored at 4 °C. The egg yolk was separated, pooled and frozen prior to IgY-*HpU* purification.

Isolation and purification of IgY-*HpU*. IgY-*HpU* was isolated and purified as previously described (Shin *et al.*, 2003). Egg yolks were separated from the whites, and the yolk preparation was mixed with an equal volume of distilled water for 30 min, followed by the addition of 0.15% (w/v) λ-carrageenan (Wako). After centrifugation at 10 000 *g* for 30 min at 20 °C, the water-soluble fraction (WSF) was collected and filtered through a Whatman no. 1 filter paper to remove solid lipid materials. The resulting IgY was further purified from the filtrate by salt precipitation with 19% (w/v) sodium sulfate followed by ultrafiltration using an ultrafiltration membrane cartridge (Millipore) with a molecular mass cut-off of 100 kDa. Purity and yield of IgY were monitored at various stages by SDS-PAGE. The IgY content was measured by absorbance at 280 nm.

Conventional ELISA (cELISA). To assess the antibody reactivity of IgY-*HpU* to *H. pylori*, we performed the cELISA; 96-well plates were coated with *H. pylori* whole-cell lysate (500 ng per well). After blocking with 1% (w/v) BSA, 100 µl of a 1 : 200 dilution of IgY-*HpU* (1 mg ml⁻¹) was added. The remaining assay steps were performed as described above for the pELISA method.

Urease activities. IgY-*HpU* (1 and 10 mg ml⁻¹) and *H. pylori* (10⁸ c.f.u. ml⁻¹) were incubated together under 10% CO₂ for 6 h at 37 °C with rotation at 50 r.p.m. Then 50 µl urea base (2% urea and 0.03% phenol red) was added and allowed to react for 30 min. Urease activity was quantified by measuring the OD₅₅₀, using a modification to the method of Fauchere & Blaser (1990). Activity was represented as percentage of control.

Statistical analysis. All data were expressed as the means ± standard deviation (SD). The statistical significance was evaluated by Student's *t*-test. *P* values of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Mapping of the epitope identified with IgY-*Hp*

We previously reported that IgY-*Hp* was immunodominantly reactive to UreA and UreB (Shin *et al.*, 2003). Using hydropathy plots (Kyte–Doolittle) that covered the entire sequence of *H. pylori* urease, we selected regions with high hydrophilicity. The reactivity of IgY-*Hp* to each synthetic peptide fragment derived from UreA and UreB is shown in Fig. 1. Among the five synthetic peptide fragments, only UB-3 (0.41 ± 0.02) showed a significant response to IgY-*Hp*, as determined by pELISA. Fifteen amino acid residues of UreA (UA-1) showed a higher titre (0.26 ± 0.02) than three of the other synthetic peptide fragments, which were similar to that of BSA (0.12 ± 0.01) (negative control). However, we decided to use only the antigen with the strongest reactivity, UB-3, for further study. The epitopic peptide that showed a predominant response was identified as amino acid residues 396–410 (UB-3; DNDNFRKRYLSKYT) of the large subunit of *H. pylori* urease, UreB.

Treatment of *H. pylori* infection by oral administration of active antibodies specific to *H. pylori* may have merit. This hypothesis was tested recently in our previous study (Shin *et al.*, 2002). IgY (IgY-*Hp*) against *H. pylori* whole-cell lysate inhibited the growth of *H. pylori* and reduced gastric inflammation in *H. pylori*-infected Mongolian gerbils. This finding suggested that IgY could be used as a novel treatment for *H. pylori*-associated gastric diseases. However, IgY produced using whole-cell lysates presents the likelihood of cross-reactivity with other bacteria, including bacteria that

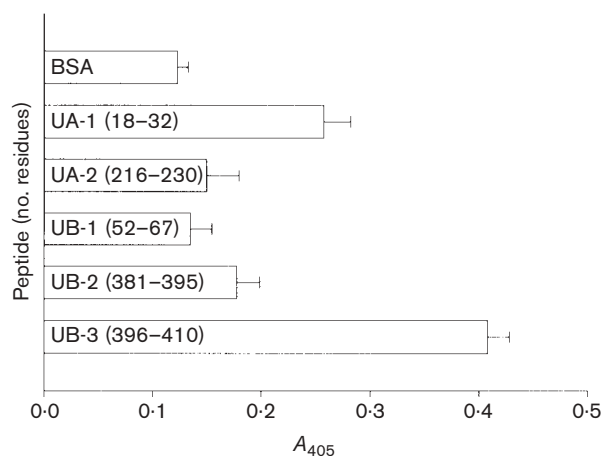


Fig. 1. Reaction between the synthesized peptide or BSA, as a negative control, and IgY-*Hp* as determined by pELISA. One hundred microlitres of a 1 : 1000 dilution of IgY-*Hp* (1 mg ml⁻¹) was added to 96-well plates coated with synthetic peptide (10 µg per well), and the titres were measured using ELISA.

are normally found in humans. To address this problem, we identified the following *H. pylori* proteins as those that reacted immunodominantly to IgY-Hp: UreA, UreB, heat-shock proteins (HSP60), probable peroxiredoxin and probable thiol peroxidase (Shin *et al.*, 2003). Of the five immunodominant proteins, urease plays a pivotal role in the pathogenesis of *H. pylori* infection by protecting the bacteria from the acid environment of the stomach, promoting colonization and inducing the production of ammonia (Bury-Mone *et al.*, 2001; Graham *et al.*, 1992). Urease also appears to be necessary for *Helicobacter* organisms to establish an infection. Urease-negative *H. pylori* mutants were unable to infect germ-free pigs (Eaton *et al.*, 1991), and a urease-negative *H. mustelae* strain was unable to infect ferrets (Andrutis *et al.*, 1995). Therefore, urease is considered one of the leading vaccine candidates (Michetti *et al.*, 1999; Hirota *et al.*, 2001; Guy *et al.*, 1998). Lee *et al.* (1999) showed that oral immunization against *H. pylori* urease significantly reduced colonization by *H. pylori* in rhesus monkeys.

In the present study, we produced *H. pylori* urease-specific IgY (IgY-HpU) using a BSA-conjugated, UreB-derived, 15-residue synthetic peptide. Using a synthetic peptide identified as an immunogen by epitope mapping has several advantages. The chief advantage is that peptides can be chemically synthesized, eliminating the purification steps that would be necessary for the production of vaccines using recombinant DNA technology (Barteling, 1988). Purification is often difficult and expensive because highly toxic bacterial compounds, such as LPS, must be removed from bacteria such as *Escherichia coli*. For this reason, peptide vaccines tend to be less expensive, purer and more stable than protein-containing subunit vaccines. In addition, using only a part of the antigenic protein eliminates undesirable immunological reactions to other parts of the protein. Antibody production requires the presence of both B-cell and T-cell epitopes within the antigen. Most large antigenic proteins contain both types of epitopes. However, when a peptide vaccine is constructed by identification and cloning of only the B-cell epitope, there is no guarantee that the peptide also contains a T-cell epitope (Barteling, 1988; Akbarzadeh *et al.*, 1999). Therefore, the peptide is usually conjugated to a large carrier protein, which supplies the T-cell epitopes. In this study, we used BSA as the conjugated protein.

Purification and characterization of IgY-HpU from egg yolk

IgY was successfully isolated from egg yolk using the λ -carrageenan method followed by ultrafiltration. The purity of the IgY obtained was 87.5% with a yield of 9.8 mg IgY (ml egg yolk)⁻¹ (Table 1). The egg-yolk proteins obtained during the purification of IgY were analysed by SDS-PAGE (data not shown).

The immunological properties of the IgY-BSA, obtained from hens immunized with BSA, and the IgY-HpU, obtained from hens immunized with the BSA-conjugated UB-3

Table 1. Summary of yield and purity of IgY from egg yolk

Preparation	Protein (mg)	IgY (mg)	Purity* (%)	Recovery (%)
Egg yolk	275†	11.3	4.1	100
WSF‡	34.2	10.5	30.1	92.9
SOS§	11.8	10.2	86.4	90.2
UF	11.2	9.8	87.5	86.7

*Purity (%) = mg IgY/mg protein \times 100.

†A 1 ml egg yolk was used.

‡Water-soluble fraction after 0.15% λ -carrageenan treatment.

§Salting out solution; created by 19% sodium sulfate treatment.

||Final preparation by ultrafiltration.

synthetic peptide fragment, were examined by cELISA. The cELISA titre values were 0.189 ± 0.01 and 0.652 ± 0.04 for IgY-BSA and IgY-HpU, respectively. These results indicated that IgY-HpU was highly specific to *H. pylori*.

Effect of IgY-HpU on urease activity of *H. pylori* in vitro

To determine the effect of IgY-HpU on the urease activity of *H. pylori*, we compared the urease activity in the presence of IgY-HpU with that in the presence of IgY-BSA. When *H. pylori* (10^8 c.f.u. ml⁻¹) was incubated with IgY-HpU (1 and 10 mg ml⁻¹) for 6 h, the urease activities of *H. pylori* were $70.1 \pm 8.5\%$ (0.64 ± 0.07) and $18.3 \pm 7.6\%$ (0.17 ± 0.06), respectively, of the control activity (0.92 ± 0.07). In our previous study, the effect of IgY-Hp was evaluated under the same conditions. The urease activities for *H. pylori* were $75.4 \pm 8.5\%$ and $15.5 \pm 7.4\%$ compared with that of the control after incubation with 1 and 10 mg IgY-Hp ml⁻¹, respectively (Shin *et al.*, 2002). Consequently, immunization using the synthetic peptide induced production of IgY-HpU with anti-urease activity very similar to that of IgY-Hp.

Hirota *et al.* (2001) demonstrated that immunization of rabbits with either a 19-residue peptide or the 8-residue minimal epitope resulted in the generation of anti-urease antibodies that were capable of inhibiting enzymic activity. In our study, IgY that was produced by immunization with *H. pylori* whole-cell lysate recognized a short linear epitope (residues 396–410; DNDNFRKRYLSKYT), and antibody specific for the linear epitope inhibited urease activity. Bittle *et al.* (1982) reported that a neutralizing antibody induced by a synthetic peptide responded much more strongly to antigen particles containing the linear epitope.

The synthetic UreB-derived peptide fragment resulted in the production of IgY-HpU that inhibited *H. pylori* urease activity, and may therefore be a tool for the prevention and treatment of *H. pylori* infections. The effect of urease activity inhibition on the adhesion properties of *H. pylori* needs to be clarified in further studies.

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