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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Immunological features and the ability of inhibitory effects on enzymatic activity of an epitope vaccine composed of cholera toxin B subunit and B cell epitope from *Helicobacter pylori* urease A subunit

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Abstract Epitope vaccine based on urease of *Helicobacter pylori* is a promising option for prophylactic and therapeutic vaccination against *H. pylori* infection. In this study, we constructed an epitope vaccine with mucosal adjuvant cholera toxin B subunit (CTB) and an epitope ($UreA_{183\text{-}203}$) of *H. pylori* urease A subunit named CTB-UA. The CTB-UA fusion protein was expressed in *Escherichia coli*, and the purified protein was used for intraperitoneal immunization experiments in BALB/c mice. The experimental results indicated that anti-CTB-UA antibody could recognize both *H. pylori* urease A subunit (UreA) and urease B subunit (UreB). Besides, the CTB-UA epitope vaccine had good immunogenicity and immunoreactivity and could induce specific neutralizing antibodies which showed effectively inhibitory effect on the enzymatic activity of *H. pylori* urease. CTB-UA is a promising molecule to be investigated as *H. pylori* vaccine antigen candidate.

Keywords Epitope vaccine · *Helicobacter pylori* · Cholera toxin B subunit · Urease A subunit · Neutralizing antibody

Introduction

Helicobacter pylori is a gram-negative spiral bacterium that infects greater than 50% of the world population and can cause a variety of diseases, including chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric lymphoma (Labenz and Borsch 1994; Blaser 1990; Uemura et al. 2001). *H. pylori* has been categorized by World Health Organization as a class I human carcinogen. The current treatment requiring multidrug regimens for *H. pylori* infection, a combination of at least two antibiotics and a proton-pump inhibitor, has a number of drawbacks including poor patient compliance, increasing antibiotic resistance, side-effects of the antibiotics, re-infection, and high cost (Cheng and Hu 2005; Boyanova et al. 2002; Kwon et al. 2010). Vaccination would be a cost-effective means to control this public health problem faced by one half of the world's population.

H. pylori urease is a polymeric enzyme that comprises two subunits, UreA (29.5 kDa) and UreB (66 kDa). *H. pylori* can produce large amounts of urease, and a proportion of *H. pylori* urease becomes associated with the bacterial surface. Urease permits *H. pylori* to maintain a constant internal and periplasmic pH to protect bacteria from acid. In addition to its role in colonization, urease might participate in tissue damage via the production of ammonia (Marshall et al. 1990; Eaton et al. 1991). The fact that the synthesis of active urease is a complex process involving many different proteins indicates that urease is an important target for prophylactic and therapeutic vaccine development.

Nonetheless, *H. pylori* urease-specific polyclonal IgG antibodies generated by immunization with purified *H. pylori* urease protein did not inhibit its enzymatic activity at all (Nagata et al. 1992). This result suggested that there might be two types of urease-specific antibodies; one may help to promote its enzymatic activity and aggravate the gastric disorder, and the other may be beneficial in inhibiting its enzymatic activity and preventing bacterial attachment to

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the gastric mucosa. Besides, it has recently been reported that poor response to the urease may favor persistence of *H. pylori* infection (Leal-Herrera et al. 1999). Therefore, it may be favorable to prevent or treat *H. pylori*-related diseases by epitope vaccine composed of carefully chosen urease epitope which can induce neutralizing antibody.

Several prophylactic and therapeutic vaccines against *H. pylori* have been developed, including whole-cell vaccine (Raghavan et al. 2002; Nystrom et al. 2006), recombinant subunit antigen vaccine (Rossi et al. 2004), and DNA vaccine, but no major breakthrough has been achieved. Epitope vaccine is a new strategy for inducing a specific immune response against *H. pylori* infection, avoiding the side effects of other unfavorable epitopes in the complete antigen. It has been reported that several epitope vaccines against *H. pylori* can afford prophylactic and therapeutic effects against *H. pylori* infection in BALB/c mice, but they are mainly composed of epitopes from UreB and induce the antibodies specific for UreB (Zhou et al. 2009; Zhao et al. 2007). A mouse monoclonal antibody (mAb), named HpU-2, showed the strongest inhibitory effect on the enzymatic activity of the urease suppressing the urease activity by 82% in 26 urease monoclonal antibodies (Nagata et al. 1992). Further research indicated that the HpU-2 mAbs mainly recognized UreA but weakly UreB and the epitope sequence recognized by HpU-2 mAb was a stretch of UreA-derived 21 amino acid residues ($UreA_{183\text{-}203}$) (Fujii et al. 2004; Hifumi et al. 2006). Therefore, we think an epitope vaccine, which can induce antibodies specific for both UreA and UreB, may be a better candidate for controlling *H. pylori* infection.

In this study, we constructed an epitope vaccine, designated CTB-UA, composed of the mucosal adjuvant CTB and a B cell epitope from UreA ($UreA_{183\text{-}203}$). The CTB-UA fusion protein was expressed in *Escherichia coli* in an active pentameric form, and its immunogenicity and immunoreactivity was evaluated by intraperitoneal immunization experiments in BALB/c mice.

Materials and methods

Bacterial strains and plasmids

The recombinant plasmid pET-CtUBE for highly expressing CtUBE was constructed by inserting the recombinant gene into *Nco*I/*Xho*I sites of the expression vector pET28a (Novagen, USA) under the control of the T7 promoter (Zhao et al. 2007). *E. coli* DH5α (ATCC 53868) was used as the host for propagating plasmids. *E. coli* BL21 (DE3), containing the T7 RNA polymerase gene under the control of the lac promoter in a lysogenic form, was used for expressing the fusion proteins.

Epitope vaccine design and plasmid construction

The theoretically optimal combination of the intra-molecule adjuvant CTB, $UreA_{183\text{-}203}$, and the linker between CTB and $UreA_{183\text{-}203}$ for the epitope vaccine was established on the basis of modeling and prediction using RANKPEP and DNA star software. The linker between CTB and $UreA_{183\text{-}203}$ was designed to be a seven-amino-acid, proline-containing segment (DPRVPSS) (Clements 1990). The fusion protein CTB-UA expression plasmid pETCUA was constructed by subcloning the *ctb* gene and $ureA_{183\text{-}203}$ gene into pET22b (Novagen, USA). Two complementary single-stranded DNA (ssDNA) sequences coding $ureA_{183\text{-}203}$ gene were synthesized: P1-$ureA_{183\text{-}203}$ (5′-CGAGCAG C A G C G T G G A A C T G A T T G A T A T T G G C G G CAACCGCCGCATTTTTGGCTTTAACGCGCTGGTG GATC-3′), containing a *Kpn*I restriction site and P2-$ureA_{183\text{-}203}$ (5′-TCGAGATCCACCAGCGC GTTAAAGCCAAAAATGCGGCGGTTGCCGCCAATAT CAATCAGTTCCACGCTGCTGCTCGGTAC-3′), containing a *Xho*I restriction site.

Fusion protein expression and purification

The fusion protein CTB-UA was expressed and purified according to the protocol performed as previous described (Zhao et al. 2007) with some modification. The recombinant vector pETCUA was transformed into *E. coli* BL21 (DE3) for expression of the recombinant protein CTB-UA. After induction with IPTG, bacteria were harvested by centrifugation. The pellets were lysed by the addition of a 0.2-fold volume of lysis solution at 37°C for 30 min. Then, the bacterial lysates were disrupted by mild sonication at 4°C for protein pattern analysis in soluble and insoluble cell fractions. The recombinant protein CTB-UA was purified by Ni^{2+}-charged column chromatography (Bio Basic Inc, Markham, Canada) according to the recommendation of the manufacturer. The obtained fusion proteins were dialyzed in 2 l of 30 mM Tris–HCl buffer (pH 8.0) in one step sufficiently. Finally, the recombinant proteins were then purified by anion-exchange chromatography using DEAE Sepharose FF (Amersham Pharmacia Biotech AB, Sweden) in binding buffer and eluted with elution buffer. After purification, the purity of the fusion peptide CTB-UA was analyzed by 12% SDS-PAGE and computer scan. The samples were dialyzed in 2 l of PBS and finally concentrated and stored at −70°C.

SDS-PAGE of the CTB-UA fusion protein and GM1-ELISA

In order to analyze the ability of the CTB-UA fusion protein to fold into pentamers, the samples of CTB-UA

fusion protein were not boiled and a sample buffer that did not contain β-mercaptoethanol was used in12% SDS-PAGE. The ability of the pentamers to bind to its cellular receptor was assessed by GM1 ganglioside enzyme-linked immunosorbent assay (GM1-ELISA). The experimental procedure was performed as previously described (Menezes et al. 2002). Briefly, ELISA plates (Corning Costar Corp, MA, USA) were coated with GM1 ganglioside (Sigma, St. Louis, USA) or bovine serum albumin (10 μg/ml, 100 μl/well) at 4°C overnight and blocked with 5% bovine serum albumin (BSA) for 2 h at 37°C. After washing three times with PBS solution containing 0.05% Tween-20 (PBST), the recombinant proteins were diluted, from 100 to 0.78 μg/ml in PBST, added to the plates, and incubated for 1 h at 37°C. Unbound proteins were removed from the plate by washing three times with PBST, and then, a proper dilution of anti-CTB polyclonal antibody (Biomade technology, Qingdao, China) was added to the plates and incubated for 1 h at 37°C. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, USA) was added, and the plate was incubated at 37°C for 1 h. The color reaction based on Tetramethylbenzidine (TMB, TianGen Biotech, Beijing, China) was terminated after incubation for 10 min at room temperature by the addition of 50 μl of H_2SO_4 (2 M), and the absorbance at 450 nm was measured by microplate reader.

Immunization and sample collection

Specific pathogen-free (SPF) male BALB/c mice or SD rats, 5 to 6 weeks old, were purchased from Comparative Medicine Center of Yangzhou University. All animal experiments were approved by the Animal Ethical and Experimental Committee of China Pharmaceutical University.

SPF BALB/c mice or SD rats were randomized into four groups (five mice or rats in each group) and were respectively immunized with 100 or 200 μg of the purified CTB-UA, recombinant cholera toxin B subunit (rCTB), recombinant UreB protein (rUreB), and PBS. The fusion proteins in PBS were emulsified with an equal volume of complete Freund′s adjuvant (Sigma, St. Louis, USA) for the first vaccination and with incomplete Freund adjuvant (Sigma, St. Louis, USA) for the second and third vaccinations. The fusion proteins in PBS without adjuvant were for the last booster vaccination. Antisera were separated on the fifth day after the last booster.

Western blot

Purified recombinant protein CTB-UA, rCTB, recombinant UreA protein (rUreA, Linc-Bio, Shanghai, China), and rUreB were applied to 12% SDS-PAGE under denaturing conditions and electro-transferred on to a nitrocellulose membrane at 80 mA for 2 h. Nonspecific binding sites were blocked overnight at 4°C in blocking buffer. Membranes were washed three times for 10 min with PBST and further incubated with a 1:1,000 dilution of mouse polyclonal anti-CTB-UA serum, anti-rCTB serum or normal mouse serum at 37°C for 1 h. After being washed three times with PBST, the membrane was incubated in HRP-conjugated goat anti-mouse IgG (1:10,000) at 37°C for 2 h. The protein bands were visualized with enhanced chemiluminescence detection system (Amersham, UK).

Assessment of antigen-specific antibody responses

Antigen-specific antibodies were measured by an ELISA assay. Briefly, ELISA plates were coated with 0.5 μg/well of natural *H. pylori* urease (Linc-Bio, Shanghai, China), rUreA, or rUreB at 4°C overnight. The plates were washed with PBST, and blocked with 5% (*w*/*v*) BSA in PBS. The plates were then washed with PBST and incubated with 100 μl of mouse or rat sera, serially diluted in PBS at 37°C for 1 h. After washing, HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rat IgG (General Bioscience Corporation, USA) was added, and the plates were incubated again for 1 h. The color reaction based on TMB was terminated after incubation for 10 min at room temperature by the addition of 50 μl of H_2SO_4 (2 M), and the absorbance at 450 nm was measured by microplate reader. Serum samples from mice and rats were assayed in triplicate.

Assessment of epitope peptide-specific antibody response

The epitope peptides of SVELIDIGGNRRIFGFNALVD ($UreA_{183\text{-}203}$) were synthesized commercially (TASH, Shanghai, China) by the Fmoc solid-phase method. The synthesized peptides were purified and analyzed by reverse-phase HPLC, and then, the purified peptides were identified by use of a mass spectrometer. The purity of the epitope peptide was 95.27%. The peptides were coated overnight on an ELISA plate at 4°C (10 μg/ml, 100 μl/well) and the subsequent steps of the assay were performed as described above for the indirect ELISA. All assays were performed in triplicate.

Urease inhibition assay by fusion protein-specific polyclonal antibody

A urease neutralization test was performed as previously described (Fujii et al. 2004). Briefly, the purified natural *H. pylori* urease (2 μg in 50 μl) was pre-incubated with 50 μl serial dilutions of antiserum from different groups in 96-well microtiter plates overnight at

4°C. After pre-incubation, 100 μl of the above reaction solution was mixed with 100 μl of 50 mM phosphate buffer (pH 6.8) containing 500 mM urea, 0.02% phenol red, and 0.1 mM dithiothreitol (DTT). The plates were incubated at 37°C. Color development was measured at 550 nm at 30 min intervals over a period of 3 h. Percentage inhibition was determined by the following equation:

$$[(\text{activitywithoutantiserum} - \text{activitywithantiserum})/(\text{activitywithoutantiserum})] \times 100.$$

Statistical analyses

All the statistical analyses were performed with the GraphPad Prism 5 software. Data were expressed as mean ± standard deviation (S.D). Statistical significance was tested using Student's paired *t* test. $p<0.05$ was considered as statistically significant. $*p<0.05$, $**p<0.01$, and $***p<0.001$ are considered as not significant.

Results

Design of epitope vaccine

In order to retain the immunological function of the UreA epitope ($UreA_{183\text{-}203}$) and further enhance the immunogenicity of $UreA_{183\text{-}203}$, we constructed a CTB-UA fusion protein with scientific and reasonable structure on the basis of prediction and modeling using RANKPEP and DNAstar software. The protein structure of the epitope vaccine CTB-UA is shown in Fig. 1. The UreA epitope ($UreA_{183\text{-}203}$) was designed to fuse with the C-terminal of CTB and a seven-amino-acid, proline-containing segment (DPRVPSS) was used as a spacer at linkage sites between CTB and $UreA_{183\text{-}203}$ to decrease the interaction between them. The construction process of recombinant plasmid pETCUA containing CTB-UA fusion gene is shown in Fig. 2.

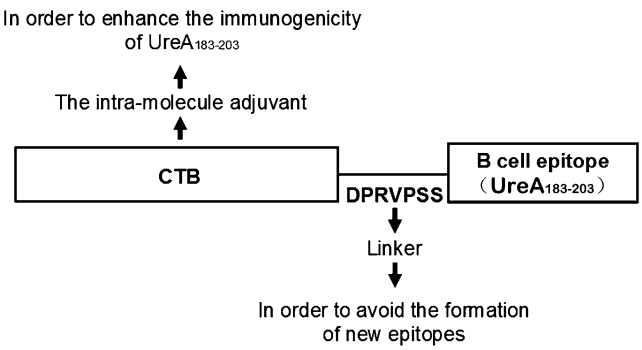

Fig. 1 Schematic representation of the designed epitope vaccine CTB-UA. CTB was selected as the intra-mucosal adjuvant to increase the immunogenic activity of the B cell epitope $UreA_{183\text{-}203}$. In order to retain the immune function of the B cell epitope $UreA_{183\text{-}203}$, CTB and $UreA_{183\text{-}203}$ were linked with the linker (DPRVPSS) to avoid the formation of new epitopes

Soluble expression and purification of CTB-UA

The recombinant protein CTB-UA was expressed in *E. coli* BL21 (DE3) and identified by its molecular mass (16 kDa). The results showed that the fusion protein CTB-UA was mainly expressed in soluble form (Fig. 3a) and had a high level of expression (about 23% of total bacterial protein). After purification by Ni^{2+}-charged column chromatography and anion-exchange chromatography, the purity of the fusion protein CTB-UA, analyzed by 12% SDS-PAGE (Fig. 3b) and computer scan, was 94.8%.

Pentamer formation and GM1-ELISA

In order to analyze the ability of CTB-UA to form pentamers, the purified fusion protein CTB-UA was

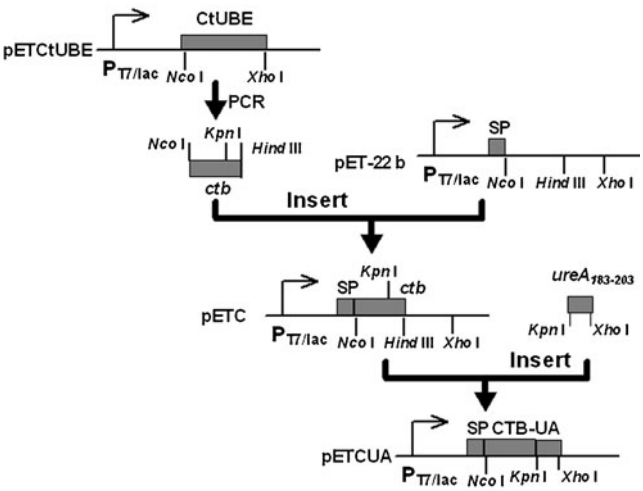

Fig. 2 Construction of the recombinant plasmid pETCUA. The recombinant plasmid pETCUA containing CTB-UA fusion gene was constructed by subcloning the *ctb* gene and $ureA_{183\text{-}203}$ gene into pET22b. The *ctb* gene was cloned by PCR amplification from pET-CtUBE. The *ctb* gene was cloned into pET22b through *Nco*I and *Hind*III restriction sites, generating the plasmid pETC. Two complementary single-stranded DNA (ssDNA) sequences coding $ureA_{183\text{-}203}$ gene were synthesized, mixed together, annealed, and inserted subsequently into the plasmid pETC through *Kpn*I and *Xho*I restriction sites, generating the recombinant plasmid pETCUA

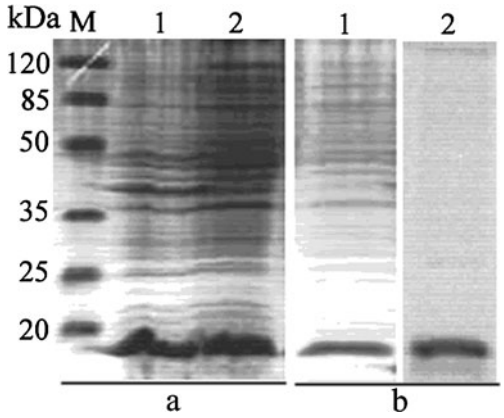

Fig. 3 The soluble expression and purification of CTB-UA analyzed by SDS-PAGE. **a** The soluble expression of CTB-UA was analyzed by 12% SDS-PAGE. *Lane 1*: the whole bacterial proteins of *E. coli* BL21 (DE3) expressing CTB-UA (16 kDa). *Lane 2*: the soluble proteins of *E. coli* BL21 (DE3) expressing CTB-UA (16 kDa). **b** Analysis of purified CTB-UA by 12% SDS-PAGE. *Lane 1*: CTB-UA purified by Ni^{2+}-charged column chromatography; *lane 2*: CTB-UA purified by DEAE sepharose FF chromatography

resuspended in sample buffer without β-mercaptoethanol and was directly loaded onto a 12% SDS-PAGE (Fig. 4a). In the reduced and boiled samples, a single band of 16 kDa (Fig. 4a, lane 1) was observed because CTB-UA could not form pentamers. However, in the non-reduced and non-boiled samples, a band of about 80 kDa (Fig. 4a, lane 2), the predicted size of the pentameric form of CTB-UA, was detected.

A GM1-ELISA was performed to confirm the ability of the CTB-UA pentamers to bind GM1. We used rCTB that was able to bind GM1 as positive control. Besides, as negative control, CTB-UA and rCTB were evaluated using BSA as the coating protein. CTB-UA and rCTB were able to bind GM1 in a dose-dependent manner (Fig. 4b). In addition to this, their curves presented the same profile. These experimental results confirmed that CTB-UA had the ability to form pentamers, and the presence of five molecules of UreA epitope peptides didn't abrogate the binding of the pentamers to its receptor.

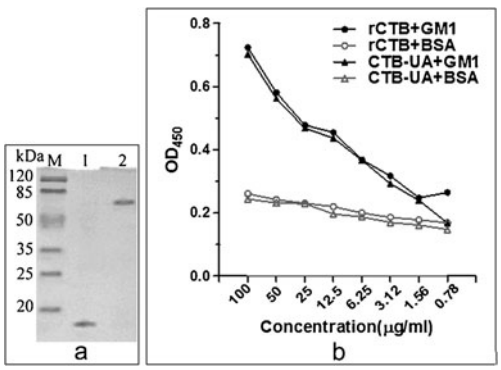

Fig. 4 Analysis of CTB-UA pentamer formation and its ability to bind GM1 receptor. **a** The fusion protein CTB-UA was loaded onto a 12% SDS-PAGE to evaluate its pentamer formation. *Lane 1*: The sample was boiled in reducing conditions. CTB-UA could not form pentamers and a single band of 16 kDa was observed. *Lane 2*: the sample was not submitted to boiling in non-reducing conditions. A band of about 80 kDa can be observed in non-boiled and non-reduced samples. **b** A GM1-ELISA was performed to confirm the ability of the CTB-UA pentamers to bind GM1. The rCTB was used as a positive control. The ELISA was performed by coating a 96-well plate with GM1 or BSA

Assessment of fusion protein-immunized mouse sera by Western blot

From a qualitative point of view, the immunogenicity and immunoreactivity of the CTB-UA fusion protein was assessed by Western blotting. Mouse anti-CTB-UA serum could react with CTB-UA, rCTB, rUreA, and rUreB specifically (Fig. 5a), while normal mouse serum did not react with any one (Fig. 5c). The results showed that the fusion protein CTB-UA had the immunological function of the UreA epitope and could induce specific antibodies against *H. pylori* urease. Moreover, mouse anti-rCTB serum could react with CTB-UA (Fig. 5b), suggesting that the CTB component was present in the fusion protein and the fusion protein did not undergo degradation.

Assessment of fusion protein-immunized mouse sera by ELISA

From a quantitative point of view, the immunogenicity and immunoreactivity of the CTB-UA fusion protein was assessed by ELISA. After immunization, serum was collected from each mouse and antigen-specific antibody levels against three antigens (natural *H. pylori* urease, rUreA, and rUreB) were measured by ELISA. A modest antibody level was observed in sera from mice immunized with the fusion protein CTB-UA. Compared with intraperitoneal immunization with rCTB or PBS, intraperitoneal

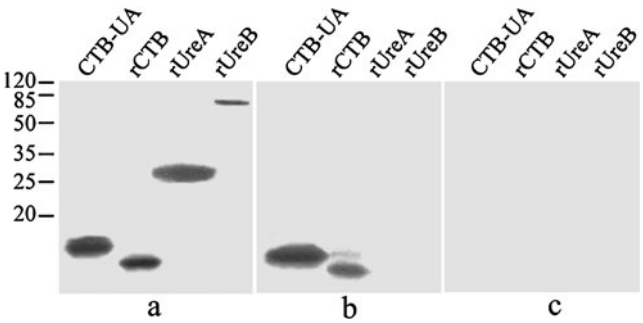

Fig. 5 Western blotting of recombinant epitope vaccine. **a** The fusion protein CTB-UA, rCTB, rUreA, and rUreB were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Mouse anti-CTB-UA serum was used for detecting immunoreactivity to rUreA and rUreB. **b** Mouse anti-CTB serum was used for detecting CTB component in CTB-UA. **c** Normal mouse serum was used as control

immunization with CTB-UA significantly increased the levels of specific IgG ($p<0.001$) against natural *H. pylori* urease, rUreA, or rUreB (Fig. 6). There was no significant difference between CTB-UA-immunized group and rUreB-immunized group on the level of specific antibodies against natural *H. pylori* urease, indicating that the fusion protein CTB-UA had good immunogenicity and immunoreactivity. In addition, the same results were obtained from the rats immunized with CTB-UA (Fig. 7), except that the specific antibody level of CTB-UA-immunized rats were lower than that of CTB-UA-immunized mice.

To examine the specific anti-peptide antibody levels elicited by the CTB-UA, the anti-$UreA_{183\text{-}203}$ antibody response was evaluated by synthesizing the $UreA_{183\text{-}203}$ peptide.

Intraperitoneal immunization with CTB-UA in both mice and rats significantly increased the levels of specific IgG ($p<0.001$) against the $UreA_{183\text{-}203}$ peptide compared with intraperitoneal immunization with rCTB, rUreB, or PBS (Figs. 6 and 7). The results indicated that anti-urease antibodies induced by CTB-UA were mainly anti-$UreA_{183\text{-}203}$ antibodies different from that triggered by rUreB.

Inhibition of urease activity by fusion protein-specific polyclonal antibody

In order to test the effect of antibodies induced by CTB-UA, a urease neutralization assay was performed. The purified natural *H. pylori* urease was pre-incubated with a serial dilution of IgG from mice immunized with CTB-UA, rUreB, or rCTB, and the inhibitory effect of the antibodies on the enzymatic activity of *H. pylori* urease was assayed. The inhibition by anti-CTB-UA polyclonal antibodies or anti-rUreB polyclonal antibodies was dose-dependent and the former is more excellent. However, anti-rCTB polyclonal antibodies didn't inhibit the enzymatic activity of *H. pylori* urease obviously (Fig. 8). This result indicated that the antibodies induced by CTB-UA have neutralization activity and the ability of anti-CTB-UA polyclonal antibodies to inhibit the urease activity was better than that of anti-rUreB polyclonal antibodies, which may be due to the presence of neutralizing antibodies against *H. pylori* urease with a higher specificity induced by the $UreA_{183\text{-}203}$ epitope within CTB-UA.

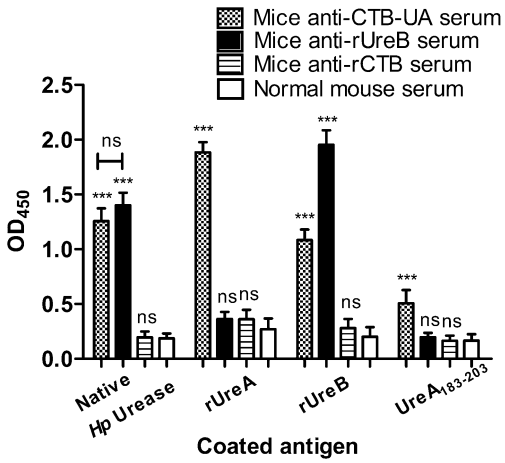

Fig. 6 Detection of specific antibodies in CTB-UA-immunized mice by ELISA. Data are expressed as mean ± S.D. The mice were immunized with CTB-UA, rCTB, rUreB, or PBS by intraperitoneal injection. Antisera samples were collected on the fifth day after the last booster. The serum was diluted 1:5,000. $p<0.05$ was considered as statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *ns* not significant

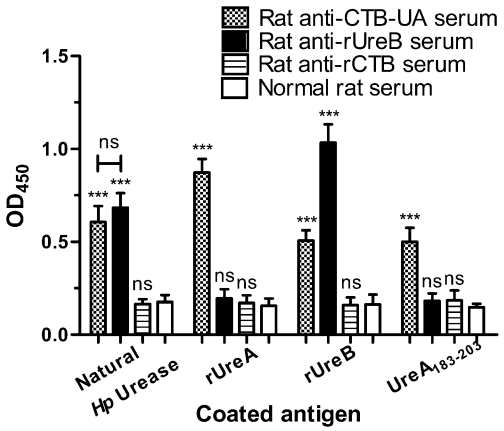

Fig. 7 Detection of specific antibodies in CTB-UA-immunized rats by ELISA. Data are expressed as mean ± S.D. The rats were immunized with CTB-UA, rCTB, rUreB, or PBS by intraperitoneal injection. Antisera samples were collected on the fifth day after the third booster. The serum was diluted 1:2,000. $p<0.05$ was considered as statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *ns* not significant

Discussion

H. pylori infection is one of the most common infections in human beings worldwide. It causes chronic gastritis, peptic

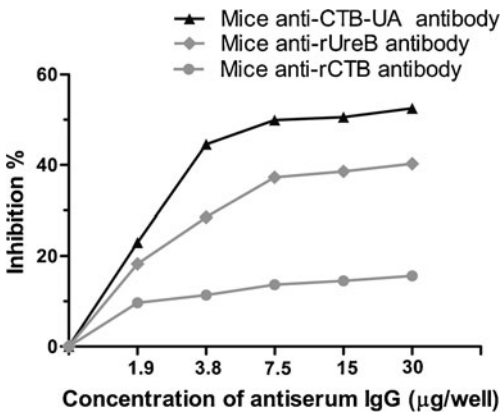

Fig. 8 Inhibition of *H. pylori* urease activity by fusion protein-specific antibody. The purified natural *H. pylori* urease was pre-incubated with a serial dilution of IgG from mice immunized with CTB-UA, rUreB, or rCTB. The optical density of the mixture was determined at 550 nm by the indicator of phenol red. The data are expressed as percentage inhibition

ulcers, and is an important risk factor for gastric cancer later in life. *H. pylori* infections cause very high morbidity and mortality, and they impose a major burden upon health care systems worldwide. Vaccination against *H. pylori*, both to prevent and to treat *H. pylori* infection, is an attractive strategy, either as an alternative or a complementary to antibiotic treatment. *H. pylori* urease is an important target for prophylactic and therapeutic vaccine development for its outstanding features. Several preventive and therapeutic vaccines based on *H. pylori* urease have been developed, being mainly genetically engineered subunit vaccines (Londono-Arcila et al. 2002; DiPetrillo et al. 1999; Wehrle et al. 1963; Bumann et al. 2001), but no major breakthrough has been achieved. Epitope vaccine can induce a specific immune response against *H. pylori* infection and had a much better specificity and security than other vaccines. Consequently, we think that an epitope vaccine, particularly based on *H. pylori* urease, may be a potential candidate for controlling *H. pylori* infection.

The design of an epitope vaccine is very important, and a number of factors have been shown to influence its overall success at inducing an immune response against the desired peptide sequence, and more importantly, to induce neutralizing antibodies against the pathogen (Liu et al. 2005; Liu et al. 2004; Yano et al. 2005). Epitopes have very low immunogenicity for their low molecular weight. How to enhance the immunogenicity of small molecule peptides is a hot and difficult problem. In order to elicit an effective immune response, it is a common practice to couple epitopes to a carrier protein. In this study, we constructed an epitope vaccine named CTB-UA composed of the mucosal adjuvant CTB and a B cell epitope from UreA. In order to decrease the interaction between CTB and $UreA_{183\text{-}203,}$ a seven-amino-acid, proline-containing segment (DPRVPSS) was used as a spacer at linkage sites between them. It has been proven that the linker (DPRVPSS) could retain the antigenicity of *E. coli* heat stable enterotoxin (ST) in the LTB-ST fusion protein (Clements 1990). ST consisting of 19 amino acid residues was similar to an epitope, so we still chose the linker (DPRVPSS) to decrease the interaction between CTB and $UreA_{183\text{-}203}$ in constructing the CTB-UA epitope vaccine. Besides, DNAstar software predicted that there was no generation of new epitopes at linkage sites between CTB and $UreA_{183\text{-}203}$. Our results showed that the CTB-UA epitope vaccine had good immunogenicity and immunoreactivity in BALB/c mice and SD rats.

CTB is a potent mucosal adjuvant and chemical conjugations with CTB have been performed using many different heterologous antigens (Sun et al. 1999; Kang et al. 2003). The pentameric form of CTB is responsible for the binding to the GM1 ganglioside, which is present on all nucleated mammalian cells and abundant on intestinal epithelial cells. The mucosal carrier and associated immunological properties of CTB are thought to be critically dependent on its pentameric structure and its ability to bind to GM1 receptors. These features facilitate the uptake of coupled antigens across the mucosal barrier and have also been found to lead to a greatly enhanced presentation of carried antigens by all antigen-presenting cells tested. The experimental results confirmed that CTB-UA fusion protein had the ability to form pentamers, and the presence of five molecules of UreA epitope peptides didn't abrogate the binding of the pentamers to its receptor.

To examine the immunogenicity and immunoreactivity of the CTB-UA epitope vaccine, many antigens were referred, including natural *H. pylori* urease, rUreA, and rUreB. Because natural *H. pylori* urease didn't contain protein impurities from *E. coli* host, it was more truthful and accurate to detect the level of specific antibody against *H. pylori* using natural *H. pylori* urease than recombined urease. The results showed that the antibodies induced by the CTB-UA epitope vaccine could react with those antigens specifically and there was no significant difference between CTB-UA-immunized group and rUreB-immunized group on the level of specific antibodies against natural *H. pylori* urease. Besides, the $UreA_{183\text{-}203}$ peptide was synthesized and used to examine the peptide-specific antibody response. The results indicated that the CTB-UA epitope vaccine was capable of generating antibodies directed specifically against the $UreA_{183\text{-}203}$ region of UreA.

Previous studies have found that some monoclonal antibodies (mAbs) against *H. pylori* urease have the ability to inhibit enzymatic activity (Qiu et al. 2010; Fujii et al. 2004; Hirota et al. 2001), whereas urease-specific polyclonal antibodies generated by immunization with purified *H. pylori* urease did not inhibit its enzymatic activity at all. Therefore, it may be favorable to prevent or treat *H. pylori*-related diseases by epitope vaccine composed of carefully chosen urease epitope which can induce neutralizing antibody. UreB is considered a potential antigen for the development of prophylactic and therapeutic vaccines against *H. pylori* infection, whereas there are more and more evidences suggesting that UreA is likewise an important target for prophylactic and therapeutic vaccine development (Hifumi et al. 2006; Lucas et al. 2001; Gomez-Duarte et al. 1998). The $UreA_{183\text{-}203}$ is a B cell epitope which is recognized by HpU-2 monoclonal antibody (mAb). It is interesting that the HpU-2 mAb can recognize both UreA and UreB, by which UreA is recognized more strongly than UreB. Moreover, HpU-2 showed the strongest inhibitory effect on the enzymatic activity of the urease suppressing the urease activity by 82% (Fujii et al. 2004). The results indicated that the

antibodies induced by CTB-UA have neutralization activity and the ability of anti-CTB-UA polyclonal antibodies to inhibit the urease activity was better than that of anti-rUreB polyclonal antibodies, which may be due to the presence of urease neutralizing antibodies with a higher specificity induced by the $UreA_{183-203}$ epitope within CTB-UA. Compared with the prophylactic or therapeutic epitope vaccine against *H. pylori* published before (Zhou et al. 2009; Zhao et al. 2007), the epitope vaccine CTB-UA exhibits several advantages. Firstly, the fusion protein CTB-UA is expressed in *E. coli* as a soluble protein, reducing protein loss during purification and renaturation. Secondly, the epitope vaccine CTB-UA can produce high levels of anti-urease antibodies compared to UreB, indicating it has good immunogenicity. Thirdly, the specific antibodies induced by CTB-UA can recognize both UreA and UreB and belong to neutralizing antibodies against *H. pylori* urease.

In conclusion, we have designed and constructed an epitope vaccine CTB-UA with good immunogenicity and immunoreactivity, which could induce specific neutralizing antibodies against UreA and UreB. Ongoing studies will evaluate the prophylactic and therapeutic effects of the epitope vaccine CTB-UA against *H. pylori* infection in BALB/c mice. This study will provide much experimental evidences for the development of epitope vaccines for human use.

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