

Full Length Research Paper

Localization of immunodominant linear B-cell epitopes of *Vibrio mimicus* outer membrane protein U (OmpU)

Ping-Ping Wang, Jun-Yu Cen, Hong-Mei Liu, Jin-Nian Li* and Ming Zhang

College of Animal Science and Technology, Anhui Agricultural University, Hefei, China.

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Outer membrane protein U (OmpU), an adhesion protein of *Vibrio mimicus*, is a good antigen, but its epitopes are still unclear. In order to locate the epitopes of OmpU protein, epitope prediction was performed using the amino acid sequence of OmpU protein of *V. mimicus* HX4 strain that was isolated from the diseased grass carp in Anhui province of China. The secondary structure, flexible regions, hydrophilicity, surface accessibility and antigenic index of the protein were analyzed using the DNASTar Protean program, and seven potential epitopes were screened. To verify the prediction, the potential epitope peptides were artificially synthesized and detected by peptide-enzyme linked immunosorbent assay (ELISA). The results show that the potential epitope peptides at amino acid positions 25 to 33, 90 to 98, 117 to 126, 173 to 180, 183 to 195, 211 to 218 and 239 to 250 were indeed immunodominant linear B-cell epitopes of the OmpU protein. Among them, the epitopes at amino acid positions 25 to 33, 90 to 98 and 239 to 250 were entirely exposed on the surface of the 3D structure model of OmpU protein, and they had a stronger immunoreactivity than others that were partially embedded inside the protein molecule.

Key words: *Vibrio mimicus*, outer membrane protein U (ompU), linear B-cell epitope, localization, enzyme-linked immunosorbent assay.

INTRODUCTION

Vibrio mimicus can cause severe ascites disease in aquatic animals (Maugeri et al., 2000; Wang et al., 2003), and it is also a causative agent of human gastroenteritis and food poisoning due to contact with contaminated water and aquatic products (Shah and Deokule, 2006; Chitov et al., 2008). Antibiotics are commonly used for controlling the ascites disease. However, antibiotic resistance of bacteria and drug residues in aquatic animals are caused by long-time use of antibiotics. These increase the frequency of therapeutic failure and present

a potential health risk for the people. Bacterial adhesion, which is mainly executed by adhesins, is prerequisite for bacteria to colonize host cell surfaces (Klemm et al., 2010). Developing adhesion antagonists based on the receptor binding domain of adhesin is very important for the prevention and treatment of ascites disease.

Thune et al. (1991) first reported that *V. mimicus* infection led to a large number of deaths of *Procambarus clarkia*. Afterwards, a lot of research have been undertaken to study the virulence factors and regulatory mechanisms of the pathogen. Outer membrane protein U (OmpU) serves as an adhesin for *V. mimicus* to invade host cells. The receptor binding domain of the protein can be used as candidate drug targets. In our previous studies, we found that OmpU protein is also a good antigen; its epitopes might be the core part for the binding domain of OmpU adhesin. Epitope, which determines antigenic specificity, is the specific chemical groups that are recognized by antibodies, B cells or T cells. The epitopes recognized and bound specifically by B-cell receptors or antibodies are defined as B-cell epitopes

*Corresponding author. E-mail: lijinnian2000@163.com. Tel: 15956967385. Fax: 86-551-5785935.

Abbreviations: PCR, Polymerase chain reaction; ELISA, enzyme linked immunosorbent assay; OmpU, outer membrane protein U; IPTG, isopropyl- β -D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MBP, maltose binding protein; OPD, o-phenylenediamine.

(Schlessinger et al., 2006). Currently, to our knowledge, there is no report about the B-cell epitopes of OmpU protein of *V. mimicus*. We screened and identified the immunodominant linear B-cell epitopes of OmpU protein of *V. mimicus* through epitope prediction followed by experimental validation. The immunoreactivity of each epitope was also analyzed, and these epitopes might be potentially useful in finding the receptor binding domain of OmpU adhesin.

MATERIALS AND METHODS

Bacterial strains, plasmid and experimental rabbits

V. mimicus HX4 strain was isolated from the diseased grass carp (*Ctenopharyngodon idellus*) in Anhui province of China and was identified with the API 20 NE system (bioMérieux, France). *V. mimicus* HX4 strain was routinely cultured in brain heart infusion broth (BHIB, Oxoid, UK). *Escherichia coli* TB1 strain and prokaryotic expression vector pMAL-c4X were purchased from New England Biolabs, Inc., and *E. coli* TB1 strain grew in the Luria-Bertani medium (LB medium, Oxoid, UK) supplemented with 100 µg/ml ampicillin when needed. Female New Zealand white rabbits weighing about 2.0 kg were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China) and were reared in a cage for seven days before immunization.

Prediction of linear B-cell epitopes of OmpU protein and peptide synthesis

The amino acid sequence of OmpU protein of *V. mimicus* HX4 strain (GenBank accession no.: DQ846743) was used for epitope prediction. The linear B-cell epitopes of OmpU protein were predicted using the DNASTar Protean program (DNASTAR, USA). The amino acid sequence of the OmpU protein was analyzed by the Chou-Fasman (Fasman, 1990) and Garnier-Robson algorithms (Garnier et al., 1978) for protein secondary structure, by the Karplus-Schulz algorithm (Karplus and Schulz, 1985) for flexible regions, by the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) for hydrophilicity, by the Emini algorithm (Emini et al., 1985) for surface probability, and by the Janeson-Wolf algorithm (Jameson and Wolf, 1988) for antigenicity index. The peptide sequences with high hydrophilicity, accessibility, flexibility and antigenicity were identified, and those located in α -helices and β -sheets were excluded according to the secondary structure features because epitopes are rarely found in such regions. Those in β -turn and random coil regions were considered as potential linear B-cell epitopes of the OmpU protein. The corresponding epitope peptides as well as a control peptide (ALAVSAAAV), which was a partial signal peptide of the OmpU protein and had very low hydrophilicity, accessibility and antigenicity index, were artificially synthesized by GenScript Biotechnology (China). The three-dimension (3D) structure of the OmpU protein was simulated using the Moe 2008 (Chemical Computing Group, Canada) and the potential B-cell epitopes were represented using the synchronize visualization program.

Construction of recombinant expression plasmid

According to the nucleotide sequence of the major antigenic domain gene of OmpU protein (*OmpUa* gene), a pair of primers was designed to amplify a 672-bp target fragment covering all the potential epitope regions. The primer sequences are as follows: 5'-

CGC GTC GAC AGT GGT GAC AAA GCG-3' (*Sal* I restriction site underlined) and 5'-CCC AAG CTT TTA ACC GTT GCT ATT ACT-3' (*Hind* III restriction site underlined). Using the primers, the *OmpUa* gene was amplified from the genomic DNA of *V. mimicus* HX4 strain by PCR. Briefly, the PCR products were purified, digested using *Sal* I and *Hind* III restriction enzymes, and ligated with the pMAL-c4x vector that had been digested by the same two enzymes. The ligation products were then transformed into *E. coli* TB1 competent cells (NEB, USA). After incubation at 37°C overnight, the recombinant plasmids were extracted and identified by PCR, double restriction enzyme digestion and sequencing. The positive recombinant plasmid was designated pMAL-c4x-OmpUa.

Protein expression and purification

A single colony of *E. coli* TB1 strain harboring the pMAL-c4x-OmpUa plasmid was picked and inoculated into LB liquid medium containing 100 µg/ml ampicillin, followed by incubation at 37°C overnight with shaking. Later, the culture was subinoculated into 200 ml of LB liquid medium containing 100 µg/ml ampicillin at a volume ratio of 1: 100 and incubated at 37°C for 3 h with shaking for enrichment. When the bacterial culture progressed in the logarithmic growth phase, isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma, USA) was added at a final concentration of 1 mM to induce the protein expression. After further culture at 37°C for 4 h with shaking, the cells were harvested by centrifugation at 12,000xg for 3 min. Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the expression of the recombinant protein. The recombinant maltose binding protein (MBP)-tagged OmpUa protein (hereafter referred as MBP-OmpUa protein) was purified using an amylose resin affinity chromatography column (NEB, USA). The purified protein was subjected to SDS-PAGE for purity analysis and to western-blot assay for immunoreactivity analysis using rabbit anti-MBP antibodies (NEB, USA). The protein concentration was measured using an UV spectrophotometer.

Preparation and purification of rabbit anti-MBP-OmpUa antibodies

By multipoint subcutaneous injections at the back, each New Zealand rabbit was immunized with 500 µl of 1 mg/ml purified MBP-OmpUa protein that was emulsified with an equal volume of biphasic emulsification adjuvant A5 (Yixing Biological Adjuvant Technology of China Animal Husbandry Industry, China). Two weeks later, the rabbits were boosted by multipoint subcutaneous injections of the same immune reagent at a dose of 250 µg of the purified MBP-OmpUa protein per rabbit. Blood samples were collected via the marginal ear vein before immunization and after 28 days post immunization, respectively. The obtained sera were titrated by the routine indirect enzyme linked immunosorbent assay (ELISA) using the purified MBP-OmpUa protein (5 µg/ml) as the coating antigen and using the 1: 7000 dilution of horse radish peroxidase (HRP)-labeled goat anti-rabbit IgG (TaKaRa, China) as the secondary antibody. The sera were purified using a HiTrap Protein G HP Column (Pharmacia, Sweden) in accordance with the manufacturer's instructions. The purified IgG protein was measured by UV spectrophotometry and titrated by the aforementioned indirect ELISA.

Peptide-ELISA

Wells of flat-bottom ELISA plates were coated with 40 µg/ml synthetic peptide (purity > 95%) in 0.1 M bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Antigen-coated wells were

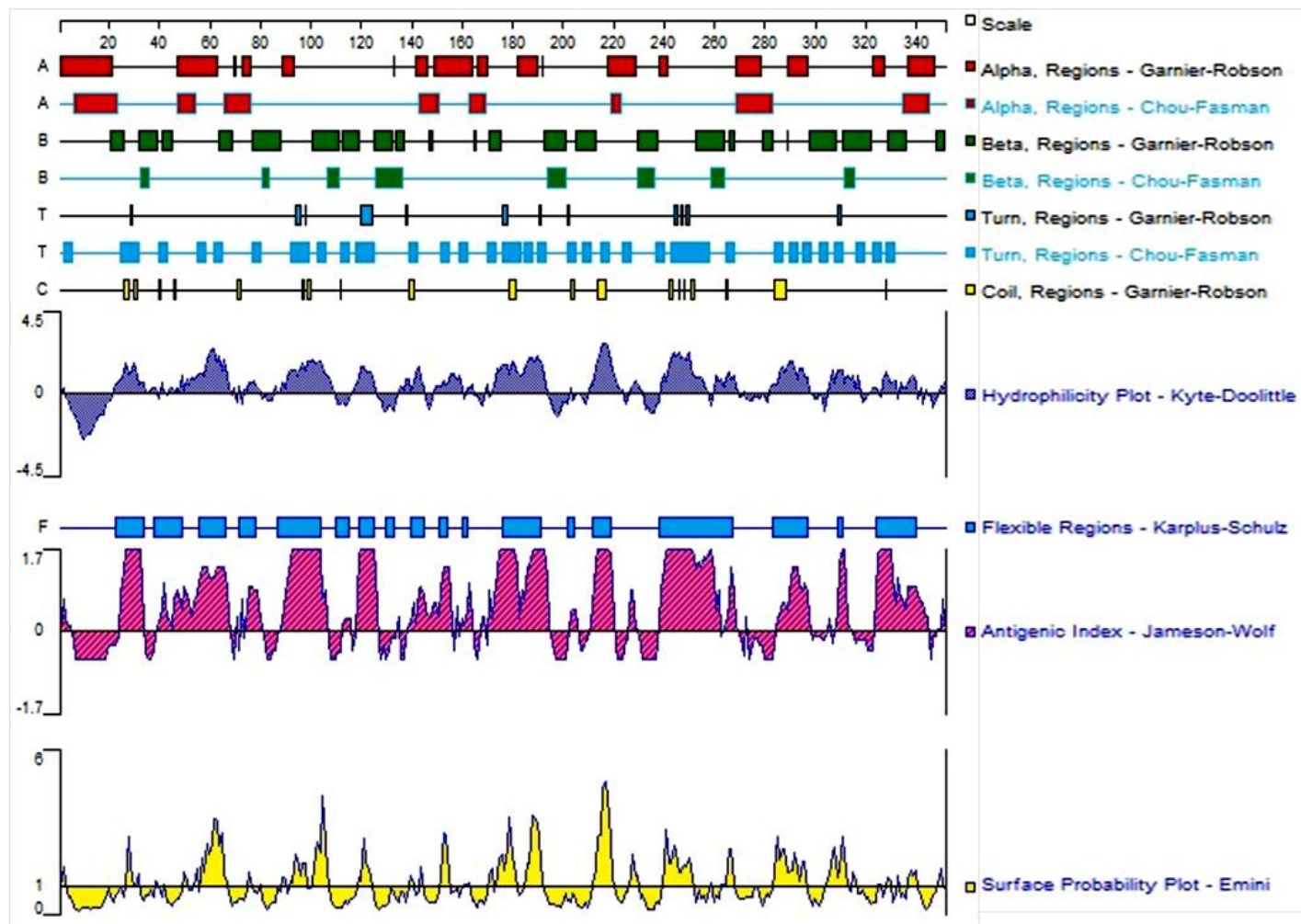


Figure 1. Epitope prediction of OmpU protein by analyzing secondary structure and other indicators (hydrophobicity, flexibility, antigenicity and surface probability) with the DNASTar Protean program.

emptied and blocked with phosphate buffered saline (PBS (pH 7.2)) containing 50 g/l nonfat powdered milk for 24 h at 4°C. After wells were emptied and rinsed one time with PBS (pH 7.2), the purified anti-MBP-OmpUa IgG and commercial rabbit anti-MBP antibodies that were 1:400 diluted in PBS (pH 7.2) were added respectively and binding was allowed to occur at 37°C for 1 h. The rabbit serum collected before immunization was used as negative control. Excess antibodies were removed by washing three times with PBS with Tween-20 (PBST), and HRP-labeled goat anti-rabbit IgG was added at a 1: 7000 dilution in PBS (pH 7.2), followed by incubation at 37°C for 1 h. Excess conjugate was washed off and o-phenylenediamine (OPD) substrate was added for color development. The enzymatic reaction was allowed to proceed in the dark for 10 min and then stopped with 1 M sulphuric acid, and the OD was measured at 490 nm in a microtiter plate-reader (Bio-Rad Benchmark Plus). The test was repeated three times. The OD₄₉₀ of rabbit anti-MBP antibodies should be less than 2.1 times that of the negative control, indicating the absence of non-specific reaction. On this premise, if the ratio of OD₄₉₀ of the purified anti-MBP-OmpUa IgG to that of the negative control ≥ 2.1 , the synthetic peptides were thought to be specifically recognized by rabbit anti-MBP-OmpUa antibodies, verifying that the predicted peptide epitopes were truly linear B-cell epitopes of the OmpU protein. The higher ratio reflected a stronger immunoreactivity of epitope.

RESULTS

Potential linear B-cell epitopes of OmpU protein and their positions in 3D protein structure model

The secondary structure, flexible regions, hydrophilicity, surface accessibility and antigenic index were predicted using the DNASTar Protean program, and the predicted results are shown in Figure 1. On this basis, seven potential immunodominant linear B-cell epitopes of the OmpU protein were selected (Table 1). These segments were located in the β -turn and random coil regions and had hydrophilic indexes ≥ 0 , surface accessibility indexes ≥ 1 and average antigen indexes ≥ 2 . Table 1 represents their positions and deduced amino acid sequences. The OmpU protein showed a very regular spatial structure. Among its potential epitopes, those at amino acid positions 25 to 33, 90 to 98 and 239 to 250 were completely exposed on the surface of 3D structure model, whereas others were embedded inside the 3D structure model at varying depths (Figure 2).

Table 1. Deduced amino acid sequences and average antigenic indexes of potential linear B-cell epitopes.

Potential epitope no.	Amino acid position ^a	Peptide sequence	Average antigenic index
1	25 to 33	NQSGDKAGS	2.51
2	90 to 98	FTTADNDSG	2.83
3	117 to 126	VTYGKNDGS	2.38
4	173 to 180	RFADRDTS	2.06
5	183 to 195	FADNKEDGY	2.49
6	211 to 218	YADQNDNN	2.02
7	239 to 250	DGEKNFDSNSNG	2.58

^aThe corresponding position of each peptide in the native OmpU protein.

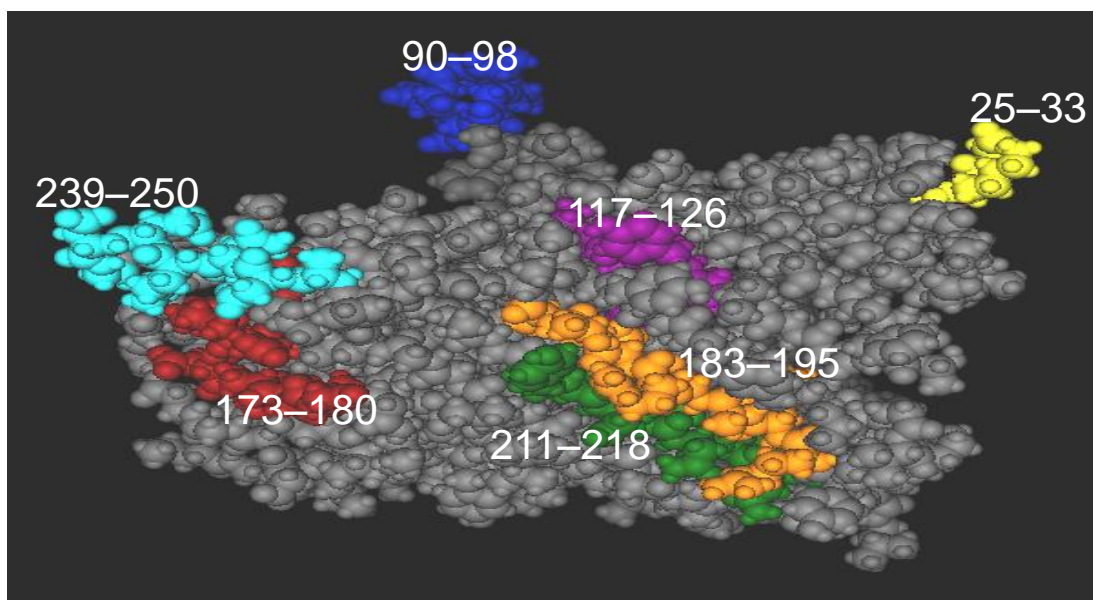


Figure 2. Visualizing potential epitopes positions in the theoretical 3D structure of the OmpU protein. The potential epitope peptides at amino acid positions 25 to 33, 90 to 98, 117 to 126, 173 to 180, 183 to 195, 211 to 218 and 239 to 250 are shown in yellow, blue, purple, crimson, saffron, green and light blue, respectively.

Obtainment of purified MBP-OmpUa protein

The SDS-PAGE analysis revealed a 66.5-kD protein band in the original and purified expression products of the pMAL-c4x-OmpUa plasmid after IPTG induction. Its size was consistent with the theoretically expected relative molecular weight of MBP-OmpUa protein (Figure 3). However, this band was not found in the total protein fractions of non-induced *E. coli* harboring the pMAL-c4x-OmpUa plasmid or pMAL-c4x vector (Figure 3a). An expectable 42.5-kD MBP protein band appeared in the whole cell lysates of IPTG-induced *E. coli* containing pMAL-c4x vector (Figure 3a). As evidenced by western-blot assay, the purified MBP-OmpUa protein could be specifically recognized by rabbit anti-MBP antibodies using the MBP protein as positive control (Figure 4). These results demonstrate the successful expression and purification of the OmpUa protein.

ELISA titer of rabbit anti-MBP-OmpUa antibodies

After two immunizations with the purified MBP-OmpUa protein, the ELISA titer of rabbit serum was $1:1.024 \times 10^6$. Using G protein affinity chromatography, 4 ml of 1.5 mg/ml pure anti-MBP-OmpUa IgG was separated from 10 ml of rabbit serum against MBP-OmpUa protein. The purified anti-MBP-OmpUa IgG had an ELISA titer of $1:4.096 \times 10^5$, which satisfied the requirements of immunoassay for antibody.

Validation and immunoreactivity of B-cell epitope peptides of OmpUa protein

Reactions between the synthetic epitope peptides and rabbit anti-MBP-OmpUa IgG were validated by peptide-ELISA. The results verify that all synthetic epitope peptides could be recognized specifically by the rabbit

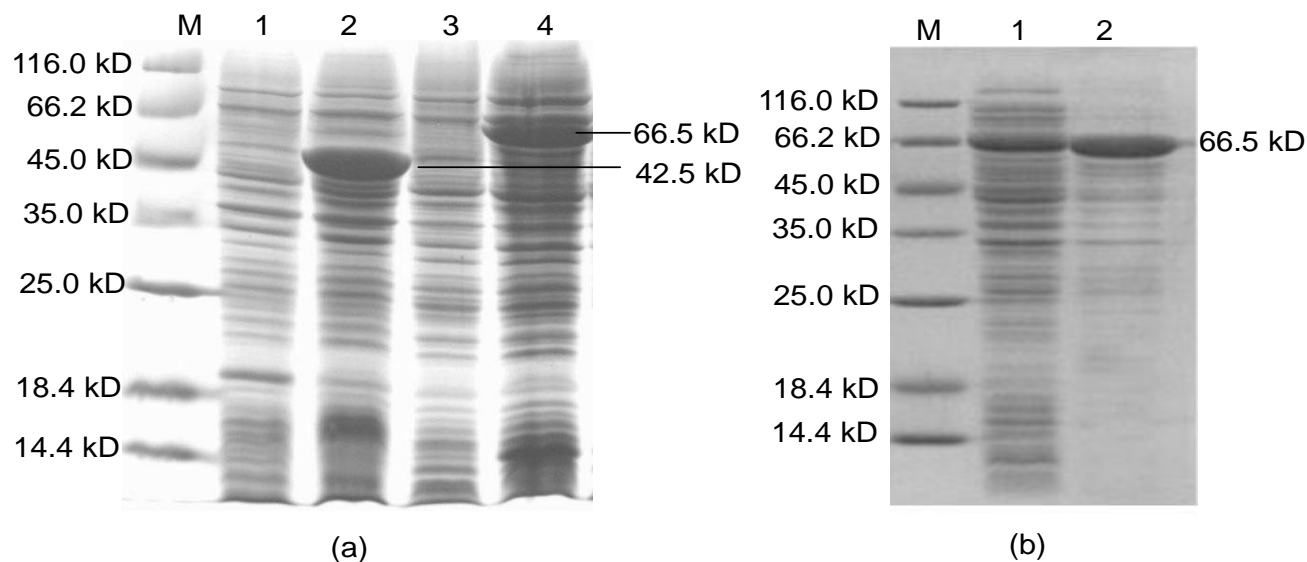


Figure 3. SDS-PAGE analysis for expression and purification of the OmpUa protein. (a) SDS-PAGE analysis for OmpUa expression in *E. coli* TB1. Cellular extracts of the non-induced and IPTG-induced *E. coli* TB1 harboring pMAL-c4x were respectively loaded on lanes 1 and 2, while those of the non-induced and IPTG-induced *E. coli* harboring pMAL-c4x-OmpUa were respectively loaded on lanes 3 and 4. The protein molecular weight standard was used to estimate the protein size (Lane M). (b) SDS-PAGE analysis for recombinant OmpUa purified by affinity chromatography. Lane 1, cellular extracts of induced *E. coli* TB1 harboring pMAL-c4x-OmpUa; Lane 2, purified recombinant MBP fusion OmpUa protein. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl- β -D-thiogalactopyranoside; OmpUa, outer membrane protein U; MBP, maltose binding protein.

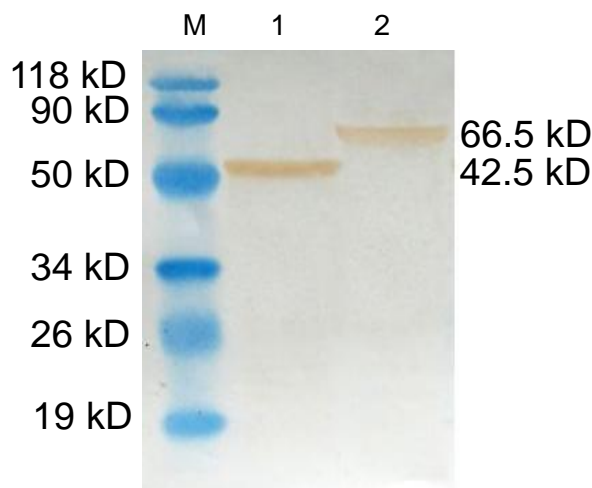


Figure 4. Western-blot analysis for recombinant MBP fusion OmpUa protein expressed in *E. coli* TB1. Commercial MBP protein (lane 1) and purified recombinant MBP fusion OmpUa protein (lane 2) were subjected to 12% SDS-PAGE and electrotransferred to nitrocellulose membrane. Then, rabbit anti-MBP antibodies were used as the primary antibody and HRP-labeled goat anti-rabbit IgG were used as the secondary antibody for the following enzymatic reaction. The prestained protein molecular weight standard was used to estimate the protein size (lane M). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OmpUa, outer membrane protein U; MBP, maltose binding protein; HRP, horse radish peroxidase.

Table 2. Immunoreactivity of the synthetic epitope peptides detected by peptide-ELISA.

Potential epitope number	Amino acid position ^a	Primary antibody ^b			Ratio ^c	
		Anti-MBP-OmpUa IgG	Anti-MBP antibody	Negative serum	Anti-MBP-OmpUa IgG	Anti-MBP antibody
1	25 to 33	0.750	0.113	0.124	6.129	0.901
2	90 to 98	0.779	0.081	0.113	6.893	0.717
3	117 to 126	0.635	0.113	0.116	5.475	0.974
4	173 to 180	0.525	0.091	0.170	3.088	0.535
5	183 to 195	0.647	0.079	0.115	5.626	0.687
6	211 to 218	0.503	0.104	0.179	2.810	0.581
7	239 to 250	0.725	0.062	0.120	6.041	0.517
8	CK	0.126	0.071	0.125	1.008	0.568

^aThe corresponding position of each peptide in the native OmpU protein; ^bthe average OD₄₉₀ value of double wells caused by the reaction between the epitope peptide and each primary antibody; ^cratio of OD₄₉₀ value of sample to that of negative serum.

anti-MBP-OmpUa IgG but could not bind with the commercial rabbit anti-MBP antibodies (Table 2). The non-specific reaction was also excluded because the control peptide could not react with the antibodies against MBP-OmpUa or MBP proteins. Therefore, binding between each epitope peptide and rabbit anti-MBP-OmpUa antibodies was specific, validating the immunoreactivity of the potential epitopes of OmpU protein. Moreover, the immunoreactivity of epitope peptides at amino acid positions 90 to 98, 25 to 33, 239 to 250, 183 to 195, 117 to 126, 173 to 180, and 211 to 218 was in a decreasing manner.

DISCUSSION

Experimental methods and prediction methods are generally used to locate linear B-cell epitopes (Wang et al., 2009). As no crystal structure is available for antigen-antibody interaction, experimental methods alone are time-consuming and laborious for the location of B-cell epitopes. For epitope prediction, bioinformatics software can reduce the experimental workload by 95% and increase location efficiency of new epitopes by 10 to 20 folds, but its accuracy is only about 75% (De Groot et al., 2002). Epitope prediction followed by concrete experimental validation is both economical and effective for epitope screening and identification. In this study, based on the characteristics of linear B-cell epitopes, we predicted the linear B-cell epitopes of OmpU protein of *V. mimicus* using the DNASTar Protean program. Seven epitope peptides were positioned and artificially synthesized. Then, we verified that they were indeed the linear B-cell epitopes of OmpU protein by peptide-ELISA.

The DNASTar Protean program is applied to predict epitopes by comprehensively analyzing many parameters such as hydrophilicity, surface accessibility, antigenic index of amino acid residues, protein secondary structure and flexible regions. Its theoretical basis is that epitopes

tend to position in peptide regions that have high hydrophilicity, surface accessibility and antigenic index. The α -helices and β -sheets, which are the secondary structure of protein, can maintain a stable structure of protein because of their high chemical bond energies and can also influence epitope location. Based on the fact that they are usually in inner parts of protein and hard to bind with antibodies, epitopes are rarely found in such regions. Nevertheless, epitopes are likely located in β -turn, random coil and flexible regions because these regions have a relatively loose structure and are easily twisted on protein surface to bind with antibodies (Lai, 1993). In our study, secondary structure, flexible regions, hydrophilicity, surface accessibility and antigenic index of OmpU protein were analyzed using the DNASTar Protean program. On the basis of the predicted results, we found seven potential immunodominant linear B-cell epitopes of the OmpU protein. All were located in β -turn, random coil and flexible regions possessed high hydrophilicity, surface accessibility and average antigenic index ($AI \geq 2$). This prediction is based on physicochemical properties of amino acid residues and characteristics of the secondary structure. However, some epitopes may be buried inside 3D structure of protein and cannot act as an antigenic determinant, leading to low prediction accuracy (Neuvirth et al., 2004).

OmpU protein of *V. mimicus* has the highest similarity with phosphoprotein. In order to obtain more accurate prediction, we simulated the 3D structure of the OmpU protein using phosphoprotein as a template by homology modeling and displayed the potential epitopes of OmpU protein on the model. The potential epitopes at amino acid positions 25 to 33, 90 to 98 and 239 to 250 were completely exposed on the surface of 3D structure model, and they had a stronger immunoreactivity than others that were partially inlaid inside the model. The relationship indicates that epitope position in the 3D structure partially determined its immunoreactivity. Our study confirms that construction of 3D structure is

necessary for screening antigenic epitopes and increases prediction reliability of linear B-cell epitopes.

Conclusion

In summary, our study identified seven immunodominant linear B-cell epitopes of OmpU protein of *V. mimicus* and these epitopes can be used to find the receptor binding domain of the protein, which is important for the development of adhesion antagonists.

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