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SYNTHESIS AND SEROLOGICAL INTERACTIONS OF *H.PYLORI* UREASE FRAGMENT 321-339 N-TERMINALLY IMMOBILIZED ON THE CELLULOSE

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Abstract: Antigenic epitopes F8 (SIKEDVQF) and UB-33 (UreB fragment with residues 321-339; CHHLDKSIKEDVQFADSRI) in *H. pylori* urease that induces neutralizing antibody production were prepared on the cellulose plate from N- to C-terminus using CDMT as a coupling reagent. Reaction of both epitopes with sera of patients with medically confirmed atherosclerosis was studied. Strong, selective reactions of both peptides with some patients sera were observed.

Keywords: *Helicobacter pylori* urease, atherosclerosis, triazine, epitope

The evidence for *Helicobacter pylori* as a gastrointestinal pathogen has steadily accumulated (1). It is one of the etiological agents of type B chronic gastritis, peptic ulcer disease and has been implicated in the development of gastric cancer (2). The most important property of *H. pylori* is its extremely high level of urease activity. The urease is very well soluble in water and is considered as a surface-layer protein of *H. pylori* making up a large part of the periplasmic proteins (3). It is suspected an important pathogenic factor because ammonia produced by urease can cause local injury to gastroduodenal mucus tissue, especially at intracellular junctions (4). Moreover, there is increasing evidence from both clinical and experimental observations that inflammation plays an important part in the pathogenesis of coronary heart disease (CHD) (5). Mendall and colleagues (6) were the first group to report a higher prevalence of *H. pylori* seropositivity in patients with CHD than in healthy volunteers.

Genetic diversity between *H. pylori* strains, which affects virulence (7) held responsibility for diversity inflammation. Specifically, strains bearing the cytotoxin associated gene A (CagA) provoke a heightened inflammatory response *in vivo* (8) and show a stronger relation with peptic ulcer disease (9) and gastric cancer (10). Pasceri and colleagues (11) observed a 3.8-fold adjusted increase in risk of CHD in *H. pylori* CagA seropositive subjects. Last five years showed vast amount of reports about connection of *H. pylori* with CHD disease (12).

Nagata et al. (13) described monoclonal antibodies (MAbs) against the native urease of *H. pylori* NCTC 11637 which inhibited the urease activity. Among the ten MAbs classified as two isotypes of the immunoglobulin G (IgG) subclass, IgGl, and IgG2a, five MAbs recognized the large subunit and the other five recognized the small subunit of the urease.

Epitope mapping methodology was applied to the derived amino acid sequences of the urease A and urease B genes of *H. pylori*. This identified 15 epitopes of which five were the most immunodominant. These were LTPKELD (Ure A), FISP, QIPTAF, EVGKVA and SIP (Ure B) (14). Takahashi and colleagues (15) identified 19 amino acid fragment of UreB (UB-33; residues from 321 to 339; CHHLDKSIKEDVQFA-DSRI) that was specifically recognized by a mouse monoclonal antibody (MAb) (L2 antibody), which were specific for *H. pylori* urease strongly inhibiting its enzymatic activity. Moreover, further sequential amino acid deletion of the 19-mer peptide from either end allowed them to determine the minimal epitope as 8 amino acid residues (F8; SIKEDVQF) for L2.

EXPERIMENTAL

Phe-OMe, Gln (Trt)-OMe, Val-OMe, Asp (OAll)-OMe, Glu (OAll)-OMe, Lys (Boc)-OMe,

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Ser (OtBu)-OMe, Ile-OMe, Arg (Pbf)-OMe, Ala-OMe, Leu-OMe, His (Trt)-OMe, Cys (Trt)-OMe were prepared from commercially available N-Fmoc amino acids by the standard procedure (16) followed be cleavage of Fmoc group with piperidine. Dibenzofulvene was removed by extraction with hexane and crude methyl esters were immediately used in the synthesis.

Abbreviations used: (Trt) – trityl; (OAll) – allyl ester; (Boc) – *tert*-butyloxycarbonyl; (tBu) – *tert*-butyl; (Pbf) – 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

Immobilization of 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) on cellulose

Whatman 7 filter paper (5 cm x 10 cm) was treated with 1M NaOH (25 mL) for 15 min and impressed to remove excess of reagent. Then solution of DCMT (1 g; 5,59 mmol) in acetone (25 mL) and solid $NaHCO_3$ (0,5 g; 5,95 mmol) was added and shaken for 50 min. The filter paper was washed with 50% acetone (25 mL), water (25 mL) and acetone (3 x 25 mL) until color reaction of filtrate with 4-(4'nitrobenzyl) pyridine (characteristic for DCMT) was not detected, then dried in the desiccator.

Attachment of the first amino acid

The cellulose plate with immobilized DCMT was treated with solution of sodium salt of amino acid (10 mmol) in water (20 mL) and shaken for 24 h. Then the plate was washed with water (3 x 25 mL) and treated with 1M aq. NH_3 (25 mL) for 40 min for cupping unreacted triazine anchoring group. The plate was again washed with water to neutralization and then treated with 1M HCl (25 mL) for 20 min. Then it was washed with water until pH=7, next washed with acetone (25 mL) for 5 min and dried in the desiccator.

The elongation of the peptide chain

Activation of carboxylic function and coupling with ester of amino acid

Dried cellulose with attached amino acid or peptide was shaken for 20 min in the solution of N-methylmorpholine (NMM) (0,27 mL; 2,5 mmol) in tetrahydrofuran (THF) (10 mL). Then an excess of NMM was removed by washing with THF (3 x 25 mL). 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (0,44 g; 2,5 mmol) in THF (10 mL) was added and the reaction was carried out for 4 h. The excess of CDMT was washed with THF (5 x 25 mL) and amino acid methyl ester (1 mmol) in N, N-dimethylformamide (DMF) (2 mL) with NMM (0,11 mL; 1 mmol) was added. The cellulose was left between glass plates for 12 h and then washed with water-isopropyl alcohol solution (1:1) (3 x 25 mL) and acetone (3 x 25 mL).

Deprotection of the carboxylic group

The plate with methyl esters was shaken for 4 h in 0.5 M LiOH (25 mL), then was washed with water until pH = 7 and treated with 1 M $NaHSO_4$ (25 mL) for 15 min. The cellulose was then washed with water to neutralization, washed with acetone and dried in the desiccator.

Cleavage of the allyl ester from side chain of Asp and Glu

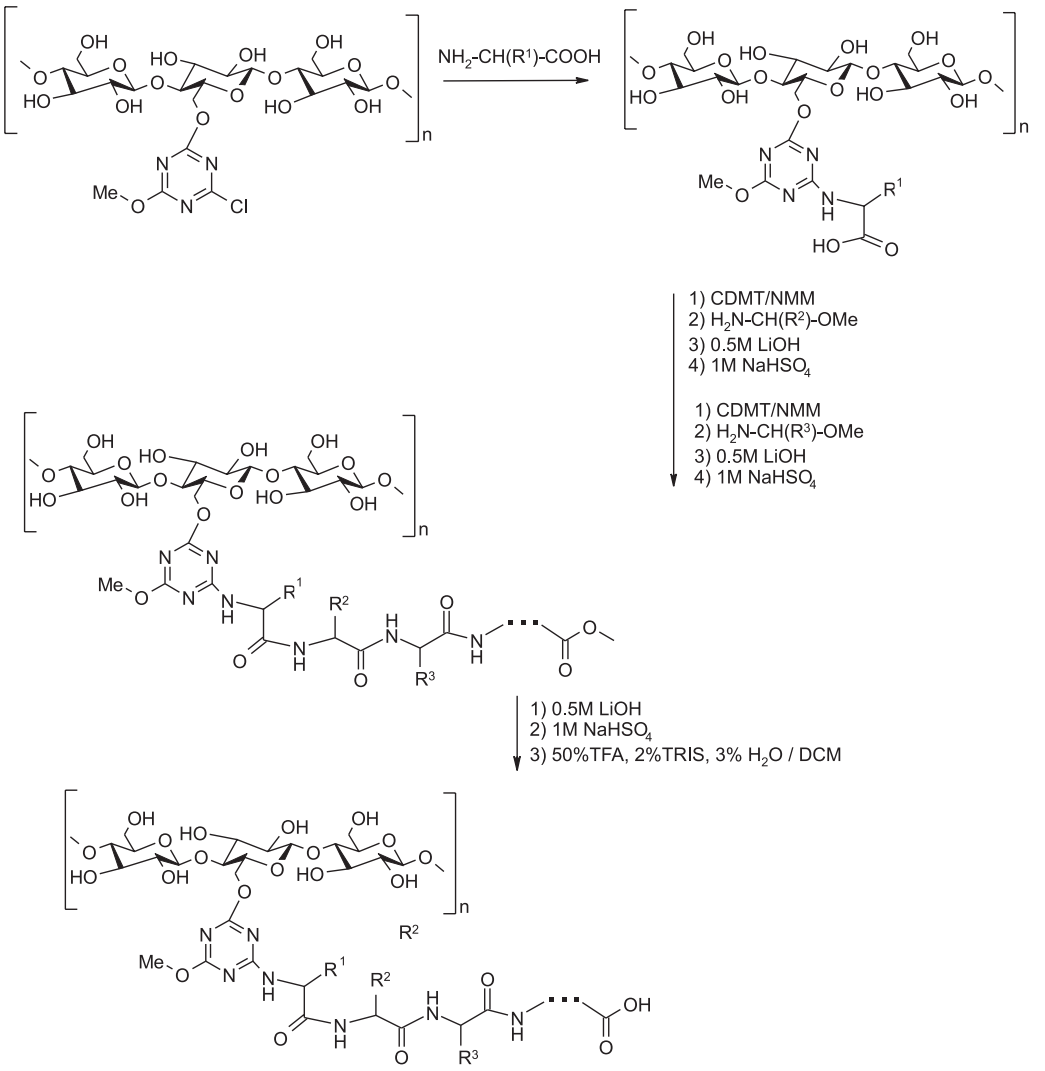
The plate with peptide was shaken under argon for 2 h in solution $CHCl_3$-AcOH-NMM (37:2:1) (10 mL, v/v/v) with Pd $(PPh_3)_4$ (1,74 g; 1,5 mmol) (17). Then the plates were washed with 0,5% N, N-diisopropyl-N-ethylamine (DIPEA) in DMF and sodium diethyldithiocarbamate (0,5%, w/w) in DMF (3 x 10 mL) to remove the catalyst and subsequently with dichloromethane (DCM) (5 x 10 mL).

Deprotection of the acid labile protecting group from the side chain of oligopeptide

The plate with peptide was treated with 20 mL solution of TFA in DCM (50%, v/v) with triisopropylsilane (TIS) (2%, v/v) and water (3%, v/v) for 4 h. Then the plates were washed with DCM (5 x 10 mL), EtOH (2 x 10 mL), DCM (2 x 10 mL) and dried in the desiccator.

Interaction with patient sera – modified Western Blot Method

Cellulose membrane with attached peptides were divided into small pieces, put into proper well of immunological 24-well or 96-well plate and treated with buffer A (Tris-NaCl, pH=7,45). In the next step it was poured into buffer B (10% fat-free instant milk dissolved in buffer A) and shaked for 120 min on a shaker. After removing buffer B, serum diluted in buffer B (1:200) (8 mL) was added. It was locked tightly and incubated at room temperature on a shaker overnight. After night, and removing sera the membrane was rinsed with buffer A six times on a shaker. After removing last drop of buffer A the cellulose membrane was poured with appropriately diluted (1:200) in buffer B class-specific (IgG) goat anti-human immunoglobulin (Sigma-Aldrich) and incubated for 120 min on a shaker. After second incubation the membrane was again rinsed with buffer A six times on a shaker. After rinsing the activity of alkaline phosphatase bound to membrane was determined by hydrolysis of 4-chloro-1-naphthol (Sigma-Aldrich) that could be



Scheme 1. Synthesis of F8 epitope (SIKEDVQF) and UB-33 epitope (CHHLDKSIKEDVQFADSRI) on the cellulose plate from N- to C-terminus using CDMT as a coupling reagent.

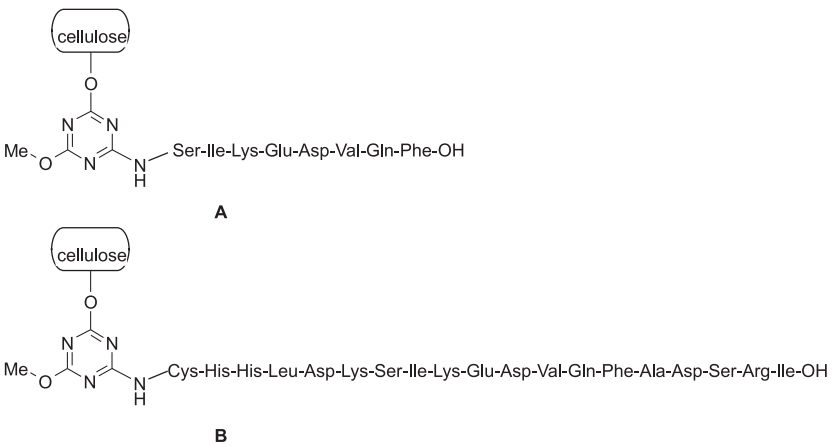


Figure 1. Structure of F8 epitope (**A**) and UB-33 epitope (**B**) attached to cellulose plate by α-amine function *via* 1,3,5-triazine scaffold.

Table 1. Reaction of peptide A, B with sera 1-6 of patients with medically confirmed atherosclerosis.

Peptide Sera	1	2	3	4	5	6
A	-	-	++	++	++	++
B	-	-	-	+++	+++	+++

visible as blue-violet color dots at place where the binding of immunoglobulin took place.

For positive and negative control experiments (by modified Western Blot Method) Jack bean urease and anti urease specific IgG antibody were used (18).

RESULTS

For our studies we have chosen antigenic epitope in *H. pylori* urease that induces neutralizing antibody production (15); F8 epitope: SIKEDVQF and UB-33 epitope: CHHLDKSIKEDVQFADSRI. These epitopes appear to lie exactly on a short sequence which formed a flap over the active site of urease, suggesting that binding of the L2 antibody sterically inhibits access of urea.

The peptides anchored *via* N–terminal moiety to the cellulose plate, were synthesized in accord to step-by-step methodology by means of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) as a coupling reagent (19). 2-Chloro-1,3,5-triazine fragment, used as an anchoring group, was introduced by the treatment of cellulose with 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) (20). The first amino acid was attached to the triazine ring in the nucleophilic substitution reaction involving amine group. The oligopeptide chain was elongated in accord to step-by-step methodology in the sequence of standard reactions involving: activation of carboxylic function, coupling with the ester of appropriate amino acid, washing, capping, hydrolysis of ester moiety, and washing (Scheme 1).

It is well known that synthesis from N-terminus to C-terminus severely increases the risk of racemization, thus potentially leading to the target compound accompanied by the family of diastereomers. In the case of massive racemization, the procedure gives the mixture of stereoisomeric peptides, which should depreciate the recognition of peptide by antibodies and in the consequence lead to unspecific reactions.

Unfortunately, classic method of enantiomeric purity determination of the peptides involving chromatography on chiral stationary phase after cleavage from the saccharide support under acidic conditions followed by subsequent degradation to amino acids is inadequate due to the fast racemization promoted by imines formed in Maillard reaction (21).

On the other hand, reports were found which documented successful synthesis and negligible racemization in case of synthesis on solid support starting from the N-terminus (22) when performed under reasonable mild reaction conditions. Therefore, we made an effort to check if methodology based on the triazine coupling reagent (Scheme 1) is resistant to the racemization sufficiently for the application in the synthesis of peptides used in the diagnostic test.

Sera used in the experiment were from patients with medically confirmed atherosclerosis (17). Every serum gave positive control test with Jack bean anti-urease antibodies. We found that 4 sera reacted with peptide **A** (short epitope) and 3 sera reacted with peptide **B** (Table 1).

DISCUSSION AND CONCLUSIONS

An amount of natural, all-L diastereomer was sufficiently abundant for selective reaction with sera 4, 5, 6 even in the case of long epitope **B**. No reaction with sera 1 and 2 and weak reaction of peptide **A** with sera 3 suggested absence of *H. pylori* urease anitibodies even if those sera gave positive results with Jack bean anti-urease antibodies. It could be caused by minor differences in amino acids compositions inside the urease fragment used in these studies. Thus, the selectivity of recognition of peptide **A** and **B** with patients sera confirmed utility of N to C methodology involving triazine coupling reagents. Further studies on the statistically representative group of atherosclerothic patients are continued.

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