Molecular Immunology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Structural and antigenic features of the synthetic SF23 peptide corresponding to the receptor binding fragment of diphtheria toxin

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ARTICLE INFO

Article history:
Received 16 February 2014
Received in revised form 1 July 2014
Accepted 5 July 2014
Available online xxx

Keywords:
Diphtheria toxin
Diphtheria toxoid
Synthetic vaccine
Affine chromatography
Fluorescence quenching
Circular dichroism

ABSTRACT

The SF23 peptide corresponding to the receptor binding fragment of diphtheria toxin (residues 508–530) has been synthesized. This fragment forming a protruding beta hairpin has been chosen because it is the less mutable B-cell epitope. Affine chromatography and ELISA show that antibodies from the sera of persons infected by toxigenic *Corynebacterium diphtheriae* and those immunized by diphtheria toxoid are able to bind the synthetic SF23 peptide. There are antibodies recognizing the SF23 peptide in the serum of horses hyperimmunized with diphtheria toxoid. Analysis of circular dichroism spectra show formation of beta hairpin by the peptide. Taken together, the results showed that the structure of the less mutable epitope of *C. diphtheriae* toxin was reproduced by the short SF23 peptide. Since antibodies against that epitope should block its interactions with cellular receptor (heparin-binding epidermal growth factor), the SF23 peptide can be considered as a promising candidate for synthetic vaccine development. Fluorescence quenching studies showed the existence of chloride and phosphate binding sites on the SF23 molecule. Phosphate containing adjuvants (aluminum hydroxyphosphate or aluminum hydroxyphosphate sulfate) are recommended to increase the SF23 immunogenic properties.

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1. Introduction

Synthetic vaccines were proposed about thirty years ago as an alternatives to vaccines produced from bacterial cultures. The first target for synthetic vaccine development was diphtheria toxin (Audibert et al., 1981, 1982). Indeed, it is much better to immunize against a small peptide than against the full length protein. A synthetic vaccine has to focus the immune system on B-cell epitopes which are important for neutralizing antibodies production. Furthermore, small peptides are safer than toxoids, even though toxoids are claimed to be completely attenuated by formalin (Metz, 2005). The risk of autoimmune reaction is decreased greatly when a short peptide with a small number of potential targets for antibody development is used. Peptide synthesis may be cheaper than toxoid production, especially if the length is less than 25 amino acid residues.

Antigenic features of diphtheria toxoid are not the same as those of diphtheria toxin. During vaccine production, diphtheria toxin is incubated for several weeks with formaldehyde (Metz, 2005). It was shown that formaldehyde forms methylol adducts and Schiff-bases with side-chains of arginine, cysteine, histidine, lysine and tryptophan residues (Metz, 2005). Moreover, formaldehyde treatment causes formation of stable cross-links between primary amino groups and several amino acid residues including arginine, asparagine, glutamine, histidine, tyrosine and tryptophan (Metz, 2005). This means that some immunogenic epitopes in some parts of toxoid molecules have different structures than those of the wild toxin. That is why a short peptide which does not require treatment with formaldehyde should be more effective than full length toxoid with partially damaged tertiary structure.

Since 1981 several peptides and even domains of diphtheria toxin were suggested as candidates for synthetic or recombinant vaccines. However, diphtheria toxoid is still used for immunization against diphtheria as a part of combined vaccines. In fact, the first suggested peptide (Audibert et al., 1981, 1982) corresponds to the loop (residues 188–201) between two alpha helices, which cannot

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http://dx.doi.org/10.1016/j.molimm.2014.07.008
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be visualized by X-ray. This loop demonstrates a rather low level of hydrophobic amino acid usage, that is not common for random coil structures situated between two alpha helices, when they are not intrinsically disordered regions (Khrustalev et al., 2013). The structure of such a "loop" peptide would be quite unstable: just a small part of peptide molecules in the given moment of time may mimic the real epitope.

Antibodies to the longer recombinant peptide (residues 168–220) did not show neutralizing activity (Lobeck et al., 1998). Moreover, this peptide had a poor capacity to bind antibodies present in equine diphtheria antitoxin (Lobeck et al., 1998). So, in our opinion, it is very important to immunize with a peptide which has quite predictable secondary (and so, tertiary) structure. The structure of the epitope should be reproducible.

It is known that some part of secondary structure elements are formed due to the contacts with other parts of a protein, which are situated close to them in a tertiary structure; while those interacting parts of a protein may be separated by dozens and hundreds of amino acid residues in a primary structure. Formation of secondary structure elements of a vaccine peptide should not require any influence of those fragments of a full length molecule which are not included into that peptide.

Immunization against the whole receptor-binding domain of diphtheria toxin was shown to be successful (Lobeck et al., 1998; Nascimento et al., 2010). However, that domain is rather long (154 amino acid residues) for the development of relatively cheap synthetic vaccine.

In the present work we report that the SF23 synthetic peptide (potential candidate for diphtheria vaccine development) created by us, passed through the first set of experiments required to test its antigenic and structural features. We selected just a single antigenic sequence of the diphtheria toxin guided by a prior *in silico* study (Khrustalev et al., 2011). Three other peptide candidates showed higher levels of mutability (see Section 4), and were excluded from *in vitro* step of the project described in the current work. The SF23 peptide corresponds to the consensus sequence of the less mutable conserved epitope of diphtheria toxin (Khrustalev et al., 2011) that has a reproducible secondary structure (beta hairpin).

According to the Epces (Liang et al., 2009) prediction, there are 13 out of 23 amino acid residues included in the SF23 peptide which are designated as antigenic ones in the structure of the full-length toxin. These residues have dark colors in Fig. 1A, unlike those residues which were considered nonantigenic (they are shown as light gray in Fig. 1). Interestingly, the Leu5 residue, considered as nonantigenic, makes a hydrophobic contact with the cellular receptor for diphtheria toxin binding (see Fig. 1B) (Louie et al., 1997). It means that the first beta strand (that includes the Leu5 residue) is situated on the surface of a protein and interacts with other proteins, including heparin-binding epidermal growth factor and, probably, with neutralizing antibodies.

In general, there are seven amino acid residues included in the SF23 peptide which are involved in different contacts with amino acid residues from heparin-binding epidermal growth factor, as it can be seen in the 1XDT (Louie et al., 1997) structure (see Fig. 1B): Leu5, Val11 and Val16 participate in hydrophobic contacts, Gln8 and Lys19 form hydrogen bonds, Lys9 and Asp12 take part in ionic interactions. So, antibodies against SF23 peptide should prevent diphtheria toxin binding to the cellular receptor if that peptide has the same or similar structure to the corresponding fragment of the full length protein.

We used affine chromatography and ELISA to show that the SF23 peptide is recognized by antibodies raised against diphtheria toxin and diphtheria toxoid. With the help of fluorescence quenching methodology we showed that the chloride ion coordination site existing in the full length molecule has been reproduced in the SF23 peptide. Circular dichroism spectrum analysis confirmed that the SF23 peptide contains beta hairpin. Those results show that the structure of the SF23 peptide is similar to the structure of the receptor binding fragment of diphtheria toxin receptor binding domain. Moreover, we showed the existence of a phosphate binding site on the peptide which should increase its adsorption on phosphate containing adjuvants.

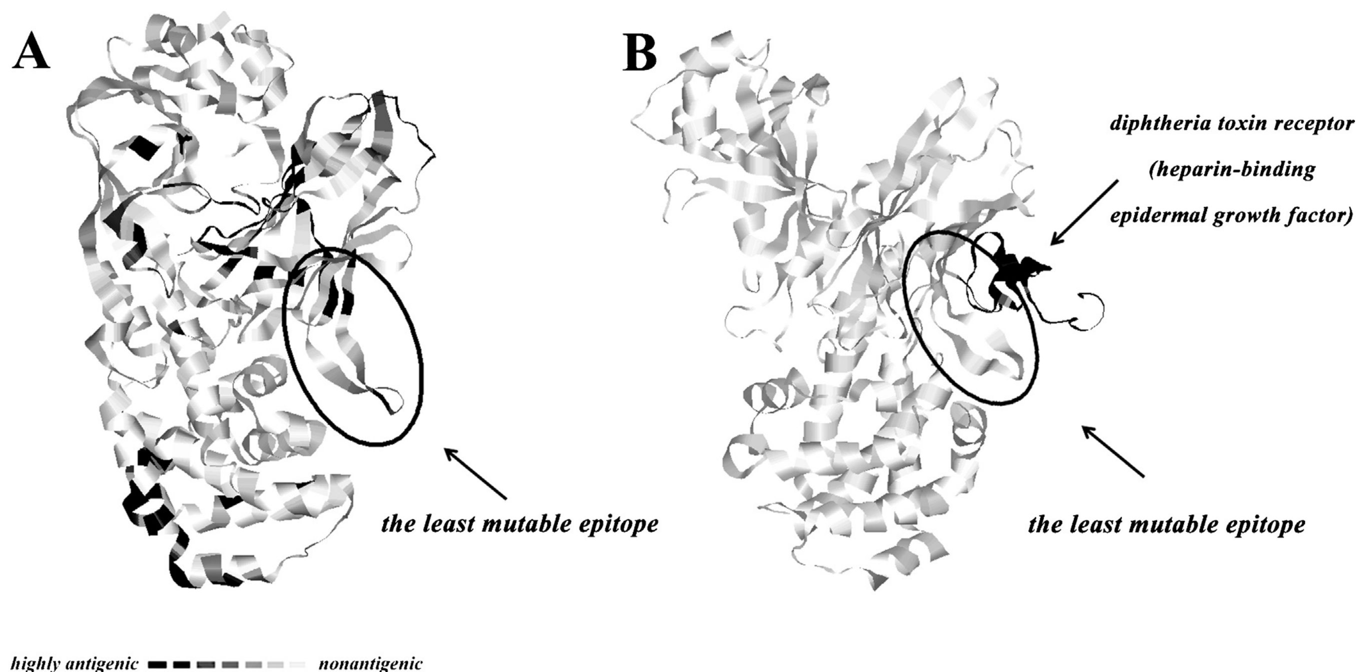

Fig. 1. Crystal structure of the diphtheria toxin (1F0L) with antigenic epitopes predicted by the Epces algorithm (A); and crystal structure of the complex (1XDT) between diphtheria toxin and its receptor (B). The epitope corresponding to the SF23 peptide is shown by the ellipse. Antigenicity level increases from light gray to black (A). Diphtheria toxin is shown in gray, its receptor (heparin-binding epidermal growth factor) is shown in black (B).

Please cite this article in press as: Khrustaleva, T.A., et al., Structural and antigenic features of the synthetic SF23 peptide corresponding to the receptor binding fragment of diphtheria toxin. Mol. Immunol. (2014), http://dx.doi.org/10.1016/j.molimm.2014.07.008

2. Materials and methods

2.1. Peptide synthesis

The SF23 peptide corresponding to the least mutable epitope of *Corynebacterium diphtheriae* toxin has been synthesized by the Peptide 2.0 Company (http://peptide20.com/). The sequence of the SF23 peptide is as follows: SIGVLGYQKTVDHTKVNSKLSLF. Automatic "Symphony" synthesizer (Protein Technologies, Inc) was used for peptide synthesis, "Shimadzu LCMS-2010" mass spectrograph and "Agilent 1200" chromatograph were used for quality control checking. Molar mass of the peptide is equal to 2535 g/mol, the level of purity is 95.4%.

2.2. Immobilization of peptide

We used "AminoLink Plus Immobilization Trial Kit" (Thermo Scientific, Inc.) to immobilize the SF23 peptide. The standard protocol of immobilization requires the usage of 0.1 M citrate buffer, pH = 10, for the first step of the procedure (Hermanson et al., 1992). The SF23 peptide cannot be dissolved in that buffer. Indeed, the isoelectric point for our synthetic peptide is somewhere between 9.539 and 10.723 according to the Isoelectric Point Calculator (http://isoelectric.ovh.org). The average pI for the peptide calculated with the usage of 10 slightly different scales of pKs for amino acids is equal to 10.012. For this reason we started the immobilization procedure at the second step, as suggested by the protocol (Hermanson et al., 1992).

The SF23 peptide (2 mg) was dissolved in 2 ml of 0.1 M PBS, pH = 7.4. Then it was added to the column and incubated with sodium cyanoborohydride (50 mM of $NaCNBH_3$) for 6 h. As a result, covalent bonds between aldehyde groups from the gel and amino groups from the peptide were formed. There are four NH_2 groups in each SF23 molecule. The next step included the quenching of remained active sites in the gel (by 1 M tris·HCl buffer pH = 7.4) in the presence of cyanoborohydride (50 mM of $NaCNBH_3$). Then the column was washed (1 M NaCl) and equilibrated with 0.1 M pH = 7.4 PBS.

2.3. Affine chromatography

The column with immobilized SF23 peptide has been used for checking the presence of antibodies able to bind the peptide in the serum of a person infected with a toxigenic *C. diphtheriae* strain, in the sera of two persons immunized with diphtheria toxoid, and in the control sera of horses which were not immunized by toxoids, as well as in the concentrated sera of horses hyperimmunized by diphtheria toxoid, *Clostridium tetani* toxoid and three types of *Clostridium botulinum* toxoids. All human participants gave written informed consent to use their sera in immunological experiments. The protocol of affinity purification included incubation of 1.5 ml of a serum dissolved in 0.5 ml of 0.1 M pH = 7.4 PBS with a gel containing immobilized SF23 molecules for 1 h, 14 washes by the 0.1 M pH = 7.4 PBS (2 ml each) and 10 washes by the IgG Elution buffer (Thermo Scientific, Inc.) which was used as the eluent (Hermanson et al., 1992).

We used a "Hitachi 650-60" spectrofluorometer to measure the intense of tryptophan fluorescence in washes and elutes (excitation wavelength = 296 nm, emission wavelength = 345 nm). Our peptide contains no tryptophan residues. So, tryptophan residues in washes and elutes are from serum proteins (including immunoglobulins) and not from the peptide itself. Levels of fluorescence in all the washes and elutes were divided by the level of fluorescence in the last wash.

We used industrially produced concentrated horse sera against *C. diphtheriae* toxoid, *C. tetani* toxoid and against *C. botulinum* toxoids type A, type B and type E, as well as control horse serum ("MicroGen" company, Russian Federation).

2.4. Enzyme-linked immunosorbent analysis

Eight wells of the high binding micro-well plate (BIOHIT, Finland) contained the SF23 peptide dissolved in 0.2 M phosphate buffer, pH = 6.9. The final concentration of the SF23 peptide was 0.01 μg per well. Control wells contained 0.2 M phosphate buffer, pH = 6.9, without peptide. After overnight incubation wells were washed 5 times (0.001 M tris·HCl with Tween 20 and NaCl, pH = 7.4) and incubated with 1% (w/v) bovine serum albumin solution in 0.2 M PBS, pH = 7.4, for 1 h at 37 °C. After the next washing procedure (see above) wells were incubated with control normal human plasma (it was reconstituted by deionized water from the lyophilized control normal human plasma produced by the Rebuplican Research and Practice Center for Hematology and Transfusiology, Minsk, Belarus) for 1 h at 37 °C. That plasma is a mixture containing material from 40 donors. The next step included washing and covering wells by anti-human IgG conjugated to alkaline phosphatase (Institute Virion\Serion GmbH, Wurzburg, Germany) for 30 min at 37 °C. Then wells were washed and filled by substrate (para-nitrophenylphosphate) solution for 30 min at 37 °C. The reaction was stopped by 1.2 M NaOH. Optical density in wells was measured with a photometer for microtiter plates at the wavelength of 405 nm. The significance of the difference between optical densities in plates covered and not covered by the SF23 peptide was checked by *t*-test.

2.5. Circular dichroism spectrography

The circular dichroism (CD) spectrum of the SF23 peptide was measured with a Jasco-815 spectropolarimeter (Jasco, Japan). The concentration of the peptide was 100 μg/ml. Measurements were performed in 0.01 M PBS, pH = 7.4, at 23 °C. All spectra were corrected against the baseline obtained with a pure buffer sample, and were smoothed using a binomial algorithm provided by Jasco. The final spectrum shows the average of five independent replicates. Scans were obtained from 300 to 190 nm at a rate of 50 nm/min with a bandwidth of 1 nm in 300 μl quartz cuvette with a path length of 1 mm. HT(V) for the spectrum studied did not exceed 487 at 190 nm. The spectra were analyzed using CDNN program (created by Dr. Gerald Böhm, Applied Photophysics Ltd., UK).

2.6. Fluorescence quenching

We used two salts (CsCl and KI) for fluorescence quenching of the SF23 peptide in the 0.1 M PBS, pH = 7.4. Manganese salts cannot be dissolved in phosphate buffer because of insoluble manganese phosphate formation. That is why we used 0.1 M tris·HCl buffer, pH = 7.4 to dissolve SF23 and four salts ($MnCl_2$, $MnSO_4$, CsCl and KI) for fluorescence quenching experiments.

In all experiments we titrated the peptide solution with 40 μl of a 1 M salt solution seven times and measured the intensity of tyrosine fluorescence (excitation wave length = 270 nm, emission wave length = 305 nm) using a "Hitachi 650-60" spectrofluorometer.

To highlight the two-phase character of the titration curves obtained, we built linear filtration trends (two neighboring points were used in that process) by MS Excel program.

2.7. Bioinformatic algorithms

We used the Epces algorithm (http://sysbio.unl.edu/EPCES/ http://sysbio.unl.edu/EPCES/) for conformational (3D) epitopes mapping (Liang et al., 2009) on the *C. diphtheriae* toxin structure (its PDB ID is 1F0L, Steere, 2001), as well as on structures of four

other bacterial toxins: *C. tetani* toxin (1AF9) (Umland et al., 1997); *C. botulinum* toxin type A (2VU9) (Stenmark et al., 2008), type B (1S0G) (Eswaramoorthy et al., 2004), and type E (3FFZ) (Kumaran et al., 2009). The Epces algorithm produces PDB files with colored residues (Liang et al., 2009). In the black and white variant of antigenicity representation used in the present work, the higher the antigenicity of a given amino acid, the stronger is the shift from light gray to black color.

The Protein Interactions Calculator (http://pic.mbu.iisc.ernet.in/) (Tina et al., 2007) has been used to study interactions between heparin-binding epidermal growth factor and diphtheria toxin on the 1XDT structure (Louie et al., 1997).

To make a model of SF23 peptide we used the Swiss Model server (http://swissmodel.expasy.org/) (Kiefer et al., 2009) in alignment mode. Peptide structure has been modeled using a 1F0L template. Even though the model of peptide and the fragment of 1F0L are very similar, there are at least two important differences introduced by the Swiss Model server. The first residue possesses a free NH_2 group, and the last residue possesses a free COOH group. Presence of free amino and carboxyl groups is very important for the correct prediction of ion binding sites.

The Isoelectric Point Calculator (http://isoelectric.ovh.org) has been used to calculate the isoelectric point for the SF23 peptide.

The BION server (http://compbio.clemson.edu/bion_server/) (Petukh et al., 2012) has been used to predict the most probable sites of Cl^- and Mn^{2+} ions binding. That server predicts ion binding sites on the basis of electrostatic interactions.

The SITEHOUND web server (http://scbx.mssm.edu/sitehound/sitehound-web/Input.html) (Hernandez et al., 2009) was used to predict the most probable sites of phosphate ions binding.

The PROSPER (protease specificity prediction server) (Song et al., 2013) has been used to predict proteolysis sites on *C. tetani* and *C. botulinum* toxins. That server (http://lightning.med.monash.edu.au/PROSPER/webserver.html) is able to predict those sites for several types of proteases and for several concrete enzymes.

3. Results

3.1. Binding of immobilized SF23 peptide by antibodies against diphtheria toxin

Concentrated horse serum containing specific antibodies is widely used for diphtheria treatment. Antibodies from that serum are able to neutralize circulating toxin. We checked the presence of antibodies able to bind the SF23 in the concentrated serum of horses hyperimmunized by diphtheria toxoid. According to the results of affine chromatography (we used AminoLink Plus column for immobilization of the peptide), there is a high peak of fluorescence intensity in the third elute (see Fig. 2A). Fluorescence intensity is directly proportional to the concentration of fluorophores. The higher the amount of tryptophan (*i.e.* the higher is the concentration of immunoglobulin), the higher is fluorescence intensity.

Antibodies able to bind the SF23 peptide have also been found by us in the serum of a toxigenic *C. diphtheriae* strain carrier, as well as in sera of persons immunized by diphtheria toxoid.

At the present time it is difficult to find persons who are not vaccinated against diphtheria. Because of this, the control horse serum ("MicroGen" company, Russian Federation) was used as a negative control. There were no peaks of fluorescence intensity after the beginning of elution (see Fig. 2B). So, there are no antibodies able to cross-react with the SF23 peptide in the serum of horses which were not immunized by diphtheria toxoid.

To show that antibodies (and not some other proteins from human or horse sera) are able to bind the SF23 peptide, we performed additional experiment with enzyme-linked immunosorbent. We adsorbed the peptide in eight wells of the BIOHIT (Finland) plate in the low concentration (0.01 μg per well) and performed standard ELISA procedure with control normal human plasma (a mixture of plasma from 40 donors produced by the Rebuplican Research and Practice Center for Hematology and Transfusiology, Minsk, Belarus), and secondary anti-human IgG antibodies conjugated with alkaline phosphatase (produced by Institute Virion\Serion GmbH, Wurzburg, Germany).

The results showed that the optical density at 405 nm in wells of microplates containing the SF23 peptide was 2.05 times higher ($P = 4.26 \times 10^{-6}$) than that in control wells which did not contain the peptide. This means that molecules of IgG from human plasma bound adsorbed peptide, while anti-human IgG antibodies conjugated with alkaline phosphatase bound molecules of human immunoglobulin. An experiment with anti-human IgG antibodies showed that the SF23 peptide is a target for immunoglobulin G binding. That IgG can be found in the blood of persons immunized with diphtheria toxin.

The least mutable epitope of diphtheria toxin is able to provoke formation of antibodies. Antibodies able to bind that epitope are produced in both human and horse organisms. The SF23 peptide should have a structure reproducible enough to bind antibodies against the corresponding epitope.

3.2. Secondary structure content of the SF23 peptide based on CD spectrum analysis

The CD spectrum represented in Fig. 3 has been analyzed by CDNN program. According to the results of that analysis, the SF23 peptide contains 34.9% of antiparallel beta sheet, 20.5% of beta turns and 34.7% of random coil. Percentages of alpha helices and parallel beta sheet are rather low. Results of CD spectrum matching with the set of reference spectra (obtained from relatively long proteins)

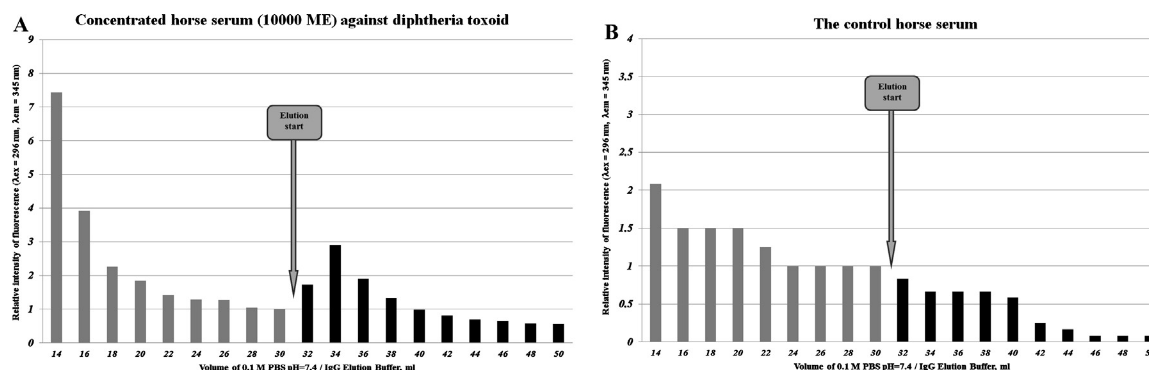

Fig. 2. Results of affine chromatography on a column with immobilized SF23 peptide for the concentrated serum of horses hyperimmunized by diphtheria toxoid (A) and the control horse serum (B).

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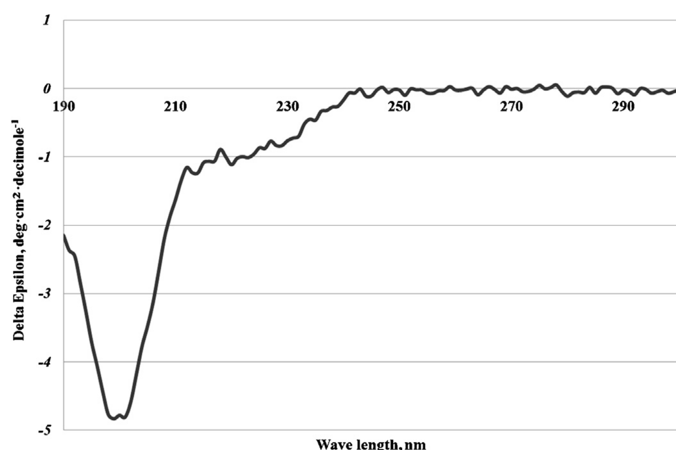

Fig. 3. Circular dichroism spectrum for the SF23 peptide dissolved in 0.01 M PBS, pH = 7.4.

should be considered on the qualitative and not on the quantitative level, at least, for short peptides. So, we may say that the SF23 peptide is a beta structural one and forms a beta hairpin (each beta hairpin should contain an antiparallel beta sheet in its stem and beta turns in its loop). We also can be sure that the SF23 peptide does not form an alpha helix and a parallel beta sheet.

3.3. Quenching of the SF23 peptide fluorescence

The structure of SF23 peptide has been tested by us with the help of fluorescence quenching as well. There is a single fluorophore (Tyr7 residue) in the SF23 peptide. Because of this, it was relatively easy to interpret the results of fluorescence quenching. In the 1F0L structure of diphtheria toxin, the chloride ion is coordinated by the nitrogen atom from the NH_2 group of Lys9 and by the nitrogen atom from the peptide bond of Lys19 residues included in the SF23 peptide (Steere, 2001). The third ligand of the same Cl^- ion (yet another nitrogen atom from the side chain of a histidine residue) is not included into the SF23 peptide.

Iodide ion should occupy the same site as chloride ion if the structure of the antigenic epitope is reproduced by the SF23. Lys19 and Lys9 can be close enough to each other if beta structure is formed by the peptide. So, the fact of I^- binding may be interpreted as additional evidence of beta structure formation by the SF23. As one can see in Fig. 4, iodide ions quench Tyr7 fluorescence much better than cationic quenchers (Cs^+ and Mn^{2+}). At the concentration of 0.038 M I^- ions occupied all available binding sites (see the point of discontinuity in the titration curve for KI from Fig. 4) and decreased the intensity of Tyr7 fluorescence to the 76.3% level (relative to the expected dilution). The distance between the coordinated I^- ion and the center of Tyr7 should be equal to 9.48 Å. In Fig. 5B, the I^- (or Cl^-) ion is shown by the gray ball.

Cesium ions are able to quench Tyr7 fluorescence only in tris·HCl buffer, while they cannot quench it in PBS (see Fig. 4). This interesting phenomenon may be caused by the phosphate binding to the cation binding site. Indeed, there is just a single site for cation binding on the SF23 predicted by the BION server (Petukh et al., 2012). That site can be found near the loop of the beta hairpin containing the only one amino acid residue with carboxyl group on its side chain (Asp12) for the whole peptide. As one can see in Fig. 5A, that loop of the SF23 hairpin is the "hottest" site for phosphate binding according to the SITEHOUND server (Hernandez et al., 2009) prediction. It is very likely that Cs^+ cation can be bound by Asp12 in tris·HCl buffer and, because of that binding, it can cause a small decrease in Tyr7 fluorescence (at the concentration of 0.051 M, Cs^+ has made the intensity of the SF23 fluorescence equal to 93.2% relative to the

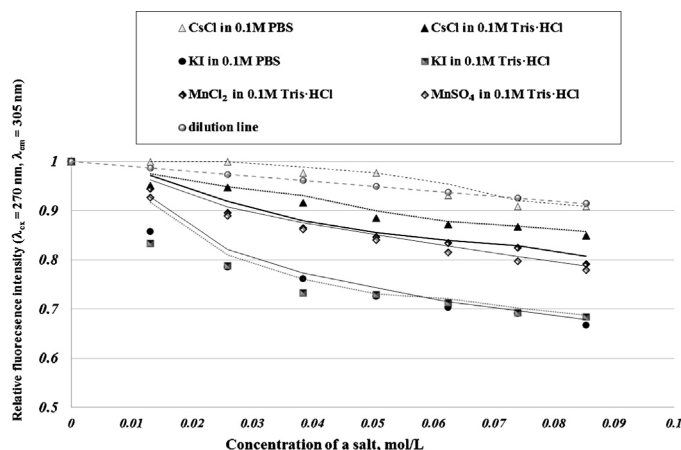

Fig. 4. Titration curves for the SF23 peptide fluorescence quenching by CsCl and KI (in 0.1 M PBS and in 0.1 M tris·HCl buffers, pH = 7.4), and by $MnCl_2$ and $MnSO_4$ (in 0.1 M tris·HCl buffer, pH = 7.4). Linear filtration trends are built by the MS Excel (two neighboring points were used).

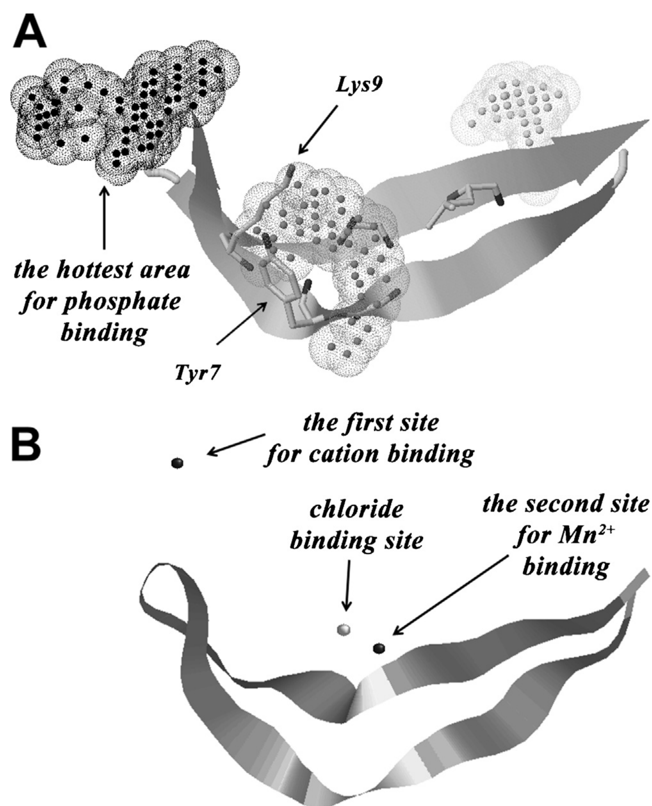

Fig. 5. 3D model of the SF23 peptide with phosphate oxygen atoms binding areas predicted by the SITEHOUND web server (A), and cation binding site predicted by the BION server, together with chloride binding site reconstructed from 1F0L structure, and suggested second Mn^{2+} binding site situated near chloride ion (B). The most probable phosphate binding area is shown by a black cloud (A). Cations are shown by black spheres, chloride ion is shown by a gray sphere (B).

expected dilution). When the experiment was performed in PBS, the phosphate ion occupied the space around the Asp12 residue and shielded the peptide from interactions with Cs^+. Here we should say that the quenching effect of Cs^+ in tris·HCl buffer is weak because of the long distance between cation bound to Asp12 and the center of Tyr7 aromatic ring (21.14 Å) (Lakowicz et al., 2002).

The manganese(II) cation can also be bound by Asp12 residue (the BION prediction supports this suggestion). In this case Mn^{2+} ions would have to quench Tyr7 fluorescence as with Cs^+ ions. In

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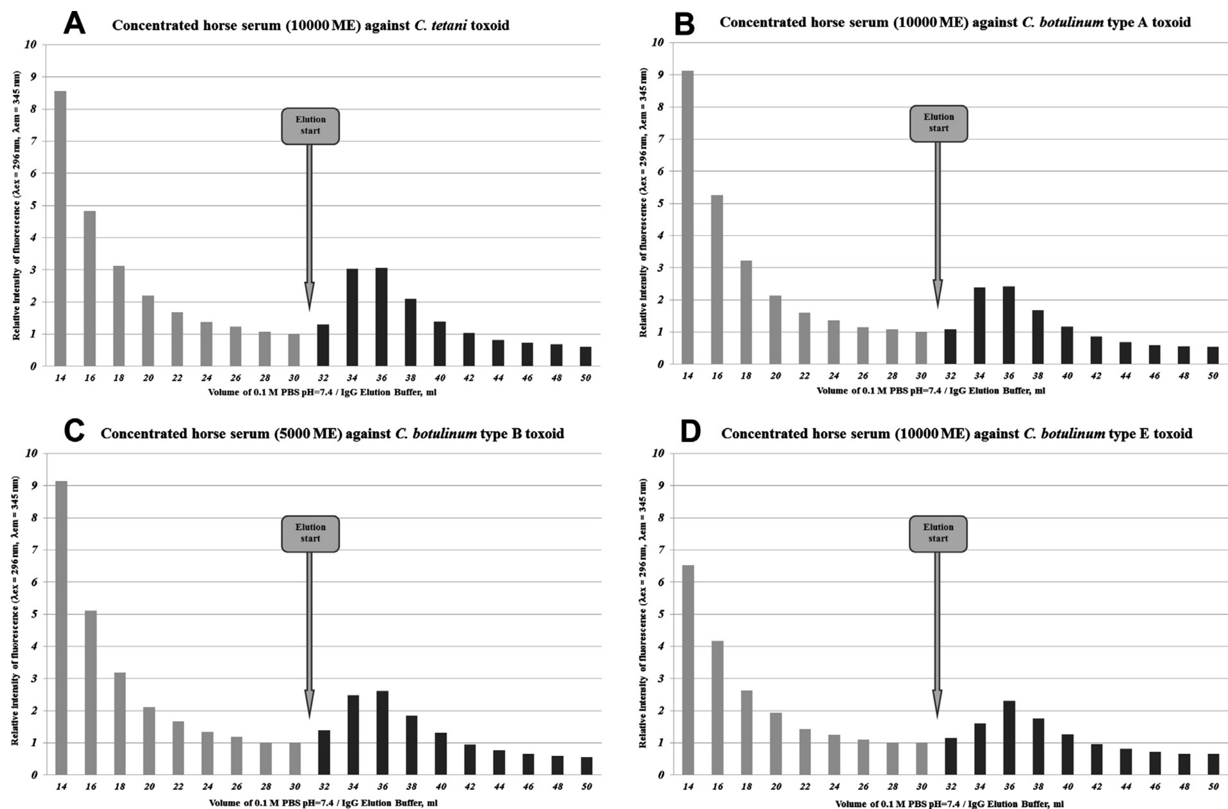

Fig. 6. Results of affine chromatography on a column with immobilized SF23 peptide for the concentrated sera of horses hyperimmunized by *C. tetani* toxoid (A), *C. botulinum* type A toxoid (B), *C. botulinum* type B toxoid (C), *C. botulinum* type E toxoid (D).

fact, Mn^{2+} ions (supplied as both $MnCl_2$ and $MnSO_4$) quench fluorescence about 1.6 times better than Cs^+ (at the concentration of 0.051 M, Mn^{2+} has made the intensity of SF23 fluorescence equal to 89.0% relative to the expected dilution). This means that Mn^{2+} ions should have at least one additional binding site. The most probable location of that site should be somewhere near already bound Cl^- ion. One of the possible coordination spheres for the second Mn^{2+} ion includes Cl^- ion, the oxygen atom from Ser18 OH group and the oxygen atom from peptide bond of Lys19. The position of that additional Mn^{2+} ion is shown in Fig. 5B by the black ball situated nearby the light gray one. On one hand, the appearance of the additional Mn^{2+} binding site on the SF23 peptide with isoelectric point equal to 10 may be explained only by the anion-dependent mechanism of binding. On the other hand, the fact of Mn^{2+} binding may be considered as yet more evidence for Cl^- coordination site existence, and so, as one more evidence of beta structure formation.

If phosphate ions from PBS are able to prevent Cs^+ interactions with the SF23 peptide, then there is a site for phosphate ion binding on it. If titration curves for manganese chloride and manganese sulfate are almost equal to each other, then sulfate ion cannot substitute chloride ion in its binding site and cannot occupy the site for phosphate binding as well. According to these results, phosphate containing adjuvants (and not those containing just sulfate anion) are preferable for future experiments on animal immunization by the SF23 peptide.

3.4. Cross-reactions of the SF23 peptide with antibodies against C. tetani and C. botulinum toxins

C. tetani and *C. botulinum* produce toxins which are similar to the *C. diphtheriae* toxin. All these toxins have three domains: beta-structural receptor-binding domain, alpha-helical "transmembrane" domain, and the catalytic domain which enters the cytoplasm (Bell and Eisenberg, 1997). The "transmembrane" domain is responsible for conformational change in lysosomes at low pH. Due to that change of conformation, the "transmembrane" domain makes a pore in the lysosome membrane and the catalytic domain enters the cytoplasm through that pore (Bell and Eisenberg, 1997). *C. tetani* toxin and different types of *C. botulinum* toxins are relatively close homologues. As to the *C. diphtheriae* toxin, it has an amino acid sequence almost completely different from those of *C. tetani* and *C. botulinum* toxins. However, similarities in the mechanism of action and in domain organization lead us to suggest that *C. diphtheriae* toxin is a very distant homologue of *C. tetani* and *C. botulinum* toxins. *C. diphtheriae* toxin has already lost most of the "similarity islands" with its distant relatives, except a single one. That last remaining "similarity island" was included in the SF23 peptide.

As one can see in Fig. 6, antibodies to *C. tetani* toxoid, as well as those to *C. botulinum* type A, B and E toxoids, can cross-react with SF23 peptide. Cross-reactions represented in Fig. 6 can be explained by the presence of an amino acid sequence in the SF23 peptide similar to those in *C. tetani* and *C. botulinum* toxins. That sequence is represented in Fig. 7. Amino acids 1–6 from the SF23 peptide (SIGVLG) form a motif which can be found in C-terminal parts of receptor-binding domains from toxins of *C. tetani* and *C. botulinum*.

Indeed, motifs similar to "SIGVLG" from the SF23 peptide can be found in four other toxins (see Fig. 7) after the alignment by the Muscle algorithm included in the MEGA 6 program (Tamura et al., 2013). In all analyzed toxins that motif exists in the form of beta strand. The first position is occupied by a hydrophilic residue (Ser, Asp, Glu or Asn). The second position contains either Ile or Leu. The fourth (Val, Phe or Leu) and the fifth (Leu, Ile or Val)

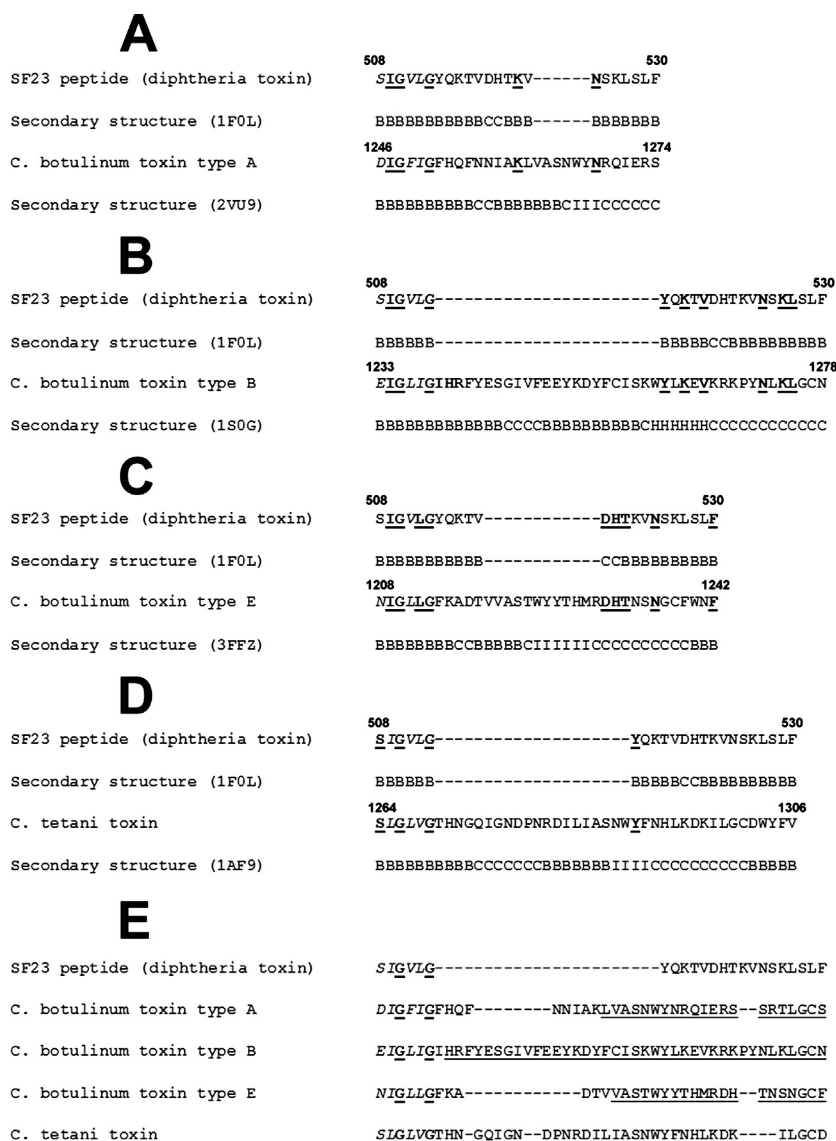

Fig. 7. Pairwise alignments of the SF23 peptide sequence with *C. botulinum* type A toxoid (A), *C. botulinum* type B toxoid (B), *C. botulinum* type E toxoid (C), *C. tetani* toxoid (D), as well as multiple alignment (E). Identical amino acid residues are in bold underlined font. Nonidentical amino acid residues from the SIGVLG motif and similar motives in *C. tetani* and *C. botulinum* toxoids are in italic font. Sequences which may be deleted due to the proteolysis according to the results of the PROSPER algorithm are in underlined font (E).

positions are also occupied by hydrophobic residues. The third and the sixth positions are occupied by Gly residues in all five toxins (see Fig. 7E).

According to the structure of the diphtheria toxin/receptor complex (1XDT) (Louie et al., 1997), the SIGVLG motif is able to interact with antibodies. In contrast, similar motifs in *C. tetani* and *C. botulinum* toxins are shielded from antibodies by their C-termini. Actually, those C-termini of toxins include helices possessing a S-X-WY motif for receptor binding (Benson et al., 2011). Those helices are situated directly on beta strands possessing motifs similar to SIGVLG from the SF23 peptide (see Fig. 8A). Proteolytic enzymes should remove C-termini of *C. botulinum* and *C. tetani* toxins (see Fig. 8B) to "open up" epitopes similar to that from diphtheria toxin (see Section 4). Anyway, according to our results, there are antibodies raised against that beta strand of those toxins and not just against their highly antigenic random coil regions. Even though epitopes made from regular secondary structure elements are thought to be less antigenic than those made from the irregular random coil, they still have sufficient antigenicity levels to provoke formation of antibodies.

4. Discussion

Epitopes used for synthetic vaccine development should be conserved and they should have low level of mutability (Khrustalev, 2010). Conservation is a term applied to evolutionary processes, while mutability is a term applied to mutagenesis. Conserved epitopes are those which are protected from amino acid substitutions by negative selection. Epitopes of a low mutability are those which are protected from amino acid substitutions by specific nucleotide usage biases in regions of DNA encoding them. In other words, the probability of missense mutation (causing amino acid replacement) should be low in regions of DNA encoding less mutable epitopes.

Theoretically, those epitopes which are conserved may be mutable: amino acid substitutions may occur fast in them, while they will

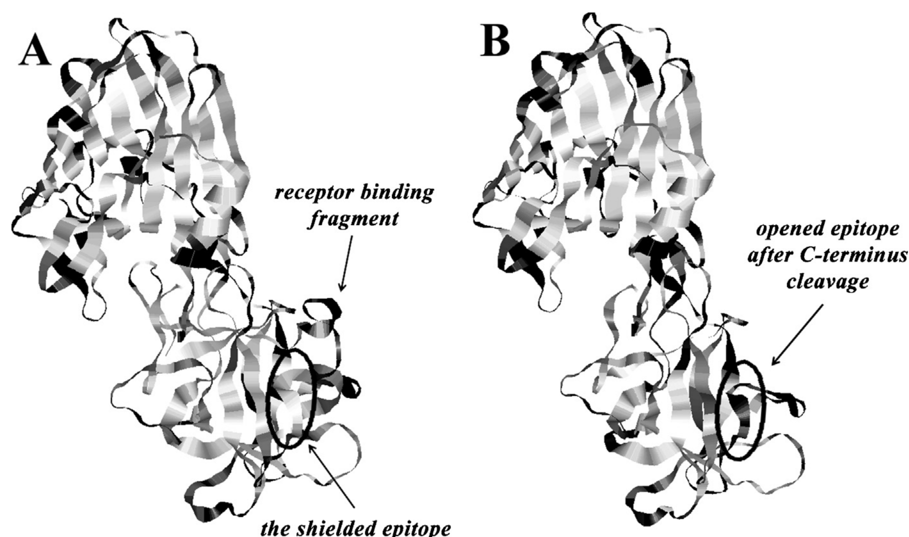

Fig. 8. Crystal structure of the full-length (A) receptor-binding domain of *C. botulinum* type A toxin (2VU9) and of its truncated form (B) with antigenic epitopes predicted by the Epces algorithm (antigenic sequences are shown by black color). Hidden (A) and opened (B) epitope similar to the SF23 peptide sequence is shown by ellipse.

be eliminated by negative selection. However, sooner or later a new substitution which has no effect on survival will appear because of the high mutability. On the other hand, an epitope with low mutability level may be quite variable because of the weak negative selection. So, a good vaccine epitope should be conserved and should have low mutability level.

According to our data, the gene coding for diphtheria toxin is prone to GC to AT mutations: there is mutational AT-pressure in that gene (Khrustalev et al., 2011). Previously we estimated the risk of missense mutation in four regions of the diphtheria toxin gene (those regions are coding for predicted antigenic B-cellular epitopes) (Khrustalev et al., 2011). As a result, we have chosen only one region with lowest probabilities of missense G to A, G to T and C to A mutations. The probability of missense G to A mutation is equal to the ratio between the usage of missense sites for G to A mutations and the sum of missense, synonymous and nonsense sites usages for that particular type of nucleotide mutation. So, the higher is the level of G in synonymous and nonsense sites for G to A transitions, and the lower is its usage in missense sites, the lower is the probability that G to A mutation in the region of DNA will cause amino acid substitution in the epitope.

For example, the sequence encoding a part of diphtheria toxin from residue 168 to residue 220 once used for peptide synthesis (Lobeck et al., 1998) has about 1.5 times higher usage of missense sites for G to A mutations than the sequence encoding the fragment from residue 508 to residue 530 corresponding to the SF23 peptide (Khrustalev et al., 2011). Since G to A transitions are frequent in the gene encoding diphtheria toxin (Khrustalev et al., 2011), the probability of amino acid substitution occurrence is higher in the 168–220 fragment than in the 508–530 region. It means that the fragment 508–530 is less mutable than the fragment 168–220.

Yet another important feature of vaccine epitope is its secondary structure. Traditionally, the most immunogenic sequences have been chosen for vaccine epitopes. Since such amino acids as Pro and Gly are usually situated on the surface of a protein, they are known as highly immunogenic ones (Hopp and Woods, 1983). The same thing can be said about tracts of hydrophilic amino acids (Hopp and Woods, 1983). Unfortunately, both sequences enriched by Pro and Gly (Chou and Fasman, 1978), as well as those with hydrophilic tracts (Khrustalev and Barkovsky, 2012), have no regular secondary structure. This means that their exact conformation depends mostly on neighboring fragments of the protein. If those irregular fragments are isolated from other parts of a protein, they will never form the same tertiary structure. Because of this, a lot of synthetic vaccine candidates failed. That is why we decided to choose a fragment of diphtheria toxin which has a regular secondary structure. As we showed in experiments on cross reaction between diphtheria toxin and toxins from *C. tetani* and *C. botulinum*, antibodies are really able to recognize beta strand of the SF23 peptide, and not just the loop between two beta strands.

It is known that some elements of secondary structure are formed because of the characteristic features of their entire amino acid sequences (Chou and Fasman, 1978). In contrast, some elements of secondary structure are formed because of the interactions with other parts of the protein. For example, one beta strand may be enriched by amino acids prone to form beta structure, while the second beta strand may have no amino acids prone to form beta structure. In that case beta structure will be formed by second beta strand just because of the presence of the first beta strand. So, vaccine epitopes should have secondary structure elements which will be reproduced because of their own features. In other words, a good vaccine epitope should not just have regular secondary structure, but it should have secondary structure elements which are predictable by methods based on amino acid propensity scales.

The chosen epitope forms a beta hairpin in the full-length toxin. That beta hairpin is formed by amino acids which are known as beta formers (Ile, Val, Tyr, Phe, Thr and Leu) (Chou and Fasman, 1978). Combinations of hydrophobic (O) and hydrophilic (W) amino acid residues in both N-terminus (WOOOO) and C-terminus (WOWOO) of the peptide sequence show high probabilities of being included in beta strands (Khrustalev and Barkovsky, 2012). So, the structure of the less mutable B-cellular epitope should have a high probability of reproduction in the synthetic peptide.

Bioinformatic methods of analyses are very useful in synthetic vaccines design. With the help of *in silico* antigenic epitopes mapping, mutability and conservation analyses, as well as with the help of secondary structure reproduction prediction one may exclude the most of the possible vaccine epitopes from the *in vitro* and *in vivo* parts of the study. Finally, just a single epitope from the whole protein should be carefully chosen on the basis of its low mutability, high conservation, sufficient antigenicity and reproducibility of its secondary structure. Such strategy helps to reduce the cost of the whole scientific project largely and to increase the quality of the future synthetic vaccine.

In the case of immunization by the full-length protein, the immune system will be overloaded by the numerous epitopes.

Some of them will be "opened up" only after the partial proteolysis of the protein. According to the results of the PROSPER algorithm (Song et al., 2013) prediction, the most probable site for matrix metallopeptidase-3 on the *C. botulinum* type A toxin is situated nine amino acid residues downstream from the end of DIGFIG motif (see Fig. 7E). Matrix metallopeptidase-3 is involved in the breakdown of extracellular proteins during tissue remodeling. That enzyme is involved in wound repair (Massova et al., 1998). So, *C. botulinum* type A toxoid should be available for matrix metallopeptidase-3 in the site of intramuscular or intradermal injection.

We modeled the protein after the cleavage by matrix metallopeptidase-3 with the help of the Swiss Model server (Kiefer et al., 2009) and predicted antigenic epitopes on that model with the help of the Epces (Liang et al., 2009). As one can see in Fig. 8B, the DIGFIG motif became more antigenic after the deletion of C-terminus. Indeed, the beta strand has a darker color in the model of truncated *C. botulinum* type A toxoid (Fig. 8B). Of course, production of antibodies against the hidden epitope is much more possible in case of hyperimmunization by the given toxoid, than in case of simple immunization. Moreover, toxoid may be partially denaturated by formalin (in that case the hidden DIGFIG motif may become exposed even before the proteolysis) (Metz, 2005).

The C-terminus of *C. botulinum* type B toxoid can be cleaved by elastase-2 according to the PROSPER algorithm prediction. Elastase-2 is secreted by neutrophils and macrophages during inflammation (Weinrauch et al., 2002). Of course, injection of any toxoid causes an inflammatory reaction. Neutrophils and macrophages are always involved not just in immunization, but also in proteolysis of antigens.

The C-terminus of *C. botulinum* type E toxoid can also be removed due to the elastase-2 activity. The C-terminus of *C. tetani* toxoid can be cleaved nearby the SIGLVG motif by both elastase-2 and matrix metallopeptidase-9.

Because of the proteolysis, the immune system deals with epitopes which can never be exposed on a functional antigen. Short peptides which do reproduce the structure of an antigenic epitope should focus the attention of immune system on the small number of targets. Moreover, only those targets which should provoke formation of neutralizing antibodies should be included in synthetic vaccine peptides.

It is known that immune response to short synthetic peptides may be relatively weak. Adjuvants are used to increase the strength of immune response. Those substances (the most commonly used adjuvants are inorganic compounds of aluminum: aluminum hydroxide, aluminum hydroxyphosphate, potassium aluminum sulfate, aluminum hydroxyphosphate sulfate, *etc.*) are thought to work by helping to retain the antigen at the injection site. It takes some time for the immune system to produce antibodies against the peptide (Caulfield et al., 2007). So, if the peptide is degraded too fast, the immune response would be insufficient. An adjuvant should have a good capacity for adsorption of a vaccine peptide. The presence of binding sites for ions included in adjuvants should increase the capacity of adsorption for a given peptide. That is why we checked the existence of phosphate and sulfate binding sites on the synthetized peptide described in the present work.

Because of the existence of phosphate binding site on the SF23 peptide, phosphate containing adjuvants should adsorb that peptide better than sulfate-containing ones.

5. Conclusions

The SF23 peptide corresponding to the less mutable B-cell epitope of the diphtheria toxin has been synthesized. The structure of the less mutable B-cell epitope has been reproduced by the SF23 peptide: antibodies against diphtheria toxin and diphtheria toxoid are able to bind immobilized SF23 peptide, according to the affine chromatography and ELISA experiments; the peptide forms a beta hairpin according to the circular dichroism spectrum analysis; there are sites for chloride and phosphate ion coordination according to the fluorescence quenching experiments.

Antibodies from concentrated serum of horses hyperimmunized against *C. tetani* and *C. botulinum* toxoids (types A, B and E) are able to cross-react with immobilized SF23 peptide because of the presence of the short common motif that becomes antigenic due to proteolysis.

The SF23 peptide can be used in future experiments needed for synthetic vaccine creation, since it has a structure similar to that of the diphtheria toxin less mutable B-cell epitope. In those experiments phosphate containing adjuvants (aluminum hydroxyphosphate or aluminum hydroxyphosphate sulfate) should be used because of the existence of a phosphate binding site on the SF23 peptide.

Acknowledgment

We thank Professor D.R. Forsdyke, Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada, for his interest in our studies, useful suggestions and significant improvements kindly introduced by him into the current report. We thank Dr. O.V. Stoma, the General Director of the "MedVax" Company, Minsk, Belarus, for his ongoing interest in our studies, support, help and sponsorship. We also thank Dr. D.G. Shcharbin, Laboratory of Proteomics, Institute of Biophysics and Cellular Engineering of the National Academy of Sciences of Belarus, Minsk, Belarus, for his help in circular dichroism experiment arrangement.

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