

Location and Reduction of Icarapin Antigenicity by Site Specific Coupling to Polyethylene Glycol

Ka-Lok Wong¹, He Li², Kin-Kwan K. Wong³, Tao Jiang² and Pang-Chui Shaw^{1,3,*}

¹State Key Laboratory of Phytochemistry and Plant Resources in West China (CUHK), Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China; ²National Key Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; ³Biochemistry Programme and Centre for Protein Science and Crystallography, School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Abstract: Icarapin is a bee venom protein found to induce IgE-mediated allergic reaction. In this study, icarapin of Asian honey bee was cloned and sequenced. By in silico screening, S198 was found to be the potential antigenic site. This site was changed to cysteine and coupled with PEG_{5K}. Compared to the wild type icarapin and the S198C variant, PEGylated S198C variant induced lower level of IgG and IgE antibodies in mice, showing that it is indeed located in an antigenic site. Our work may be generalized to other proteins for the discovery of antigenic sites and the reduction of antigenicity.

Keywords: Allergenic site, allergy, bee venom, icarapin, in silico screening, polyethylene glycol.

INTRODUCTION

Icarapin is a secretory protein found in bee venom [1]. Apart from European honey bee (*Apis mellifera*), icarapin was also found in red flour beetle (*Tribolium castaneum*) by the computer prediction of genome sequence. Icarapin has a signal peptide and potential multiple carbohydrate binding sites. The coding sequence of icarapin corresponds to a mature protein with molecular weight 22.7 kDa. This antigenic protein can induce IgE-mediated allergic reactions. Dot blotting study showed that 4 out of 5 bee venom allergic patients gave a positive signal against icarapin [2], which suggested that icarapin may be involved in the allergic response of these patients.

Allergic symptoms after bee venom injection can be very serious. The symptoms include the decrease in blood pressure, rashes on the skin, a rapid pulse to a shortness of breath, tightness of the heart, faintness and death [3]. The production of IgE antibodies against allergen can elicit strong inflammation by the activation of mast cells and basophils to release inflammatory mediators [4]. Immunotherapy using the weak recombinant allergens, synthetic peptides or fragments with reduced IgE binding capacity is now feasible [5-6]. With the help of expression cloning, it is now possible to obtain pure antigen to analyze the mechanism and recognition in allergic patients. The structural information, especially epitope, of the antigen is a prerequisite for producing recombinant allergen for the therapy [7].

The objective of this study is to find the antigenic sites of the icarapin by in silico prediction and site-specific PEGylation. This approach, which may be generalized to

other antigenic proteins, allows us to confirm that the site at S198 of icarapin is an antigenic site.

MATERIALS AND METHODS

This study could be divided into two major parts: 1) finding the potential antigenic site of the icarapin by in silico screening; 2) conducting experiment to validate the prediction (Fig. 1). In the first part, the potential antigenic site was selected according to three criteria: i) the site was on the surface of the predicted three-dimensional structure; ii) it was close to the predicted B-cell epitopes and iii) it was the O-glycosylation site. In the second part, codon at this predicted site was changed to cysteine by mutagenic PCR primers. PEG-maleimide (activated PEG) was coupled to this cysteine residue, which masked the site from the host's immune system. Wild type, variant and PEGylated variant were then used to immunize the mice. By comparing the immunogenicity of the three groups, we could prove whether the predicted site was the actual antigenic site of icarapin.

Cloning of Icarapin

Adult worker bees of Asian honey bee (*A. cerana*) were collected from Po Sang Yuen Bee Farm, Hong Kong. The whole sting apparatus of the bees were dissected out. Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA) and cDNA was synthesized by First-Strand cDNA Synthesis Kit (GE healthcare, WI, USA) according to the instructions of manufacturer. The wild-type icarapin gene was then amplified using gene specific primers which were designed according to the mRNA sequence of *A. mellifera* available in the GenBank (accession number DQ485318). DNA fragment generated by PCR was cloned into expression vector pET-24a(+) (Novagen, WI, USA) and sequenced. Amino acids positions 1-19 of the protein sequence were found to be the signal peptide by the SignalP 3.0 Server [8]. The forward primer for amplifying icarapin without signal

*Address correspondence to these authors at the State Key Laboratory of Phytochemistry and Plant Resources in West China (CUHK), Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China; Tel: +852 26096803; Fax: +852 26037246; E-mail: pcshaw@cuhk.edu.hk

The first two authors contributed equally to this work.

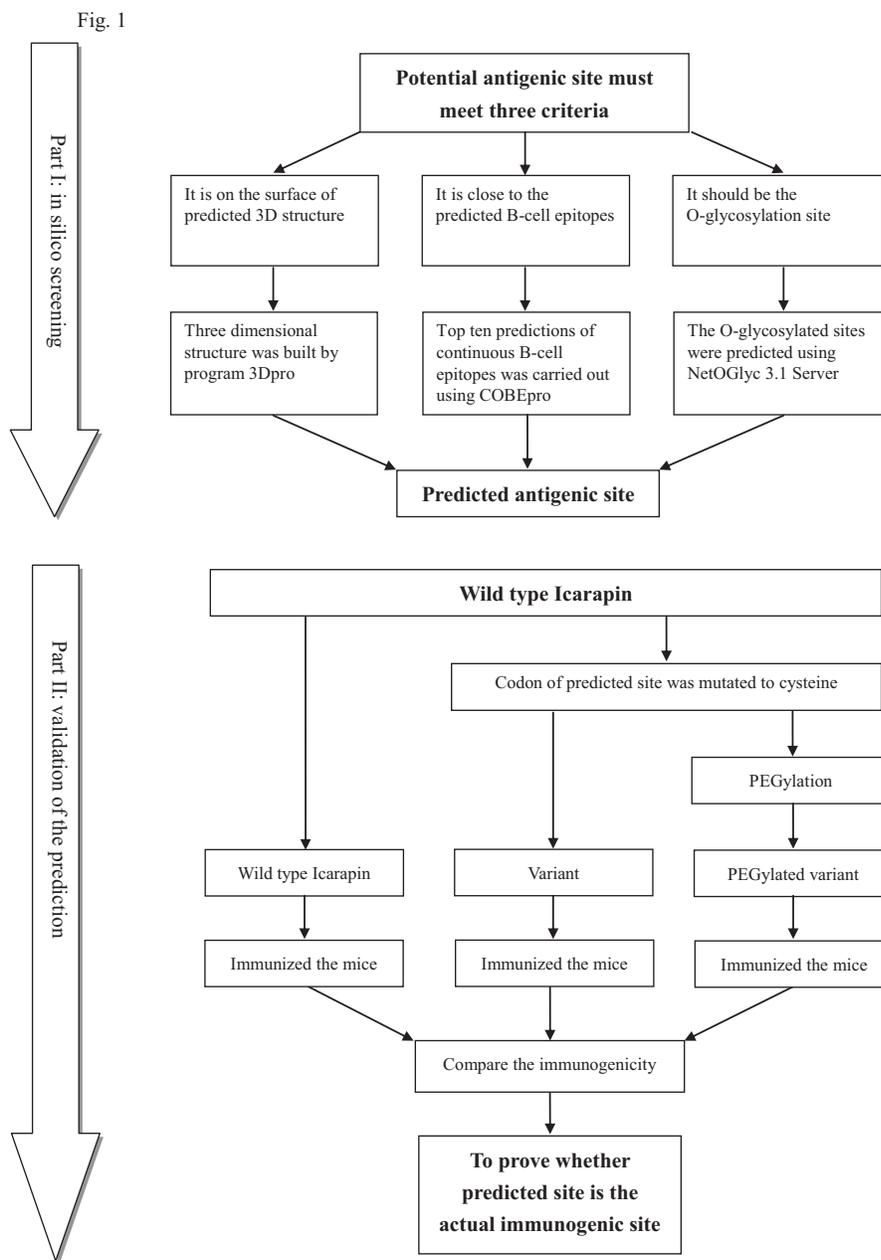


Figure 1. The workflow. The work is divided into two major parts. The first part was to find the potential antigenic sites on icarapin by in silico screening. The second part was to validate the prediction.

peptide was designed and the amplified PCR fragment was subcloned into expression vector pET-24a(+).

Selection of Mutation Site

The three-dimensional structure of wild type icarapin without signal peptide was predicted using program 3Dpro on SCRATCH Protein Predictor Server [9]. The amino acid sequence was uploaded to the server and the calculation was performed with default parameters. The prediction of top ten continuous B-cell epitopes was carried out using COBEpro [10] on SCRATCH Protein Predictor Server, and the O-glycosylated sites were predicted using NetOGlyc 3.1 Server [11]. We found that S198 (position refers to icarapin with

signal peptide) met all the three screening criteria mentioned above, therefore, this site was selected for mutation. S198C mutant was constructed by overlapping PCR and then subcloned into expression vector pET-24a(+).

Expression and Partial Purification of Icarapin and its Mutants

The expression culture was lysed by sonication with buffer A (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) and the lysate was applied to a HisTrap FF (GE healthcare, WI, USA) column (5 mL). After washing with buffer A and then 150 mM imidazole in buffer A, the protein was eluted with 300 mM imidazole in buffer A.

Modification of Icarapin Variant by PEG

PEG-maleimide reagent was synthesized by reacting a 0.01 M monomethoxypolyethylene glycol amine (PEG amine with *Mr.* 5000, Sigma, MO, USA) solution in reaction buffer (0.1 M sodium phosphate, pH 6.8) with 5-fold molar excess of 6-maleimidocaproic acyl N-hydroxy succinimide ester (EMCS, sigma) for 2 hours at room temperature. The PEG-maleimide reagent was purified on a Sephadex G-25 (GE healthcare, WI, USA) column equilibrated with H₂O and lyophilized to powder. Variant was incubated with 0.1 M DTT at 4 °C overnight to break the disulfide bond and then dialyzed twice against the reaction buffer at 4 °C for 4 hours to remove excess DTT. Reaction of S198C with PEG-maleimide was carried out in an N₂-saturated reaction buffer containing 10 mM EDTA, at a variant/PEG molar ratio of 1:5, and stirred for 4 hours at room temperature.

Purification of Icarapin and its Derivatives

Partially purified icarapin, variant and PEG-variant were further purified by electro-elution. They were first loaded into a 15% SDS-PAGE gel for separation. The gel was stained with ponceau S solution. The target band was cut out from the gel and protein was recovered using electro-elution (Bio-Rad, CA, USA). Elutes were dialyzed against water at 4 °C overnight and lyophilized to powder.

Antibody Production

The immunization of mice and blood collection were carried out at Guangdong Medical Laboratory Animal Centre. C57BL/6N inbred mice (6-8 week old) in groups of six were immunized subcutaneously at the back with 10 µg of icarapin or its derivatives in complete Freund's adjuvant on Day 0. Booster injection was given with incomplete Freund's adjuvant on Day 21. Blood samples were collected 7 days after booster injection by retrobulbar puncture. Sera of mice were stored at -20 °C until used.

Assay for Immunogenicity: IgG and IgE Responses

Specific IgG and IgE antibodies against icarapin and its derivatives were detected by indirect ELISA. ELISA plates (Maxisorp immunoplate, Thermo Fisher Scientific, MA, USA) were coated overnight at 4 °C with 80 µL of antigen (0.125 µg) in 0.05 M sodium carbonate/bicarbonate buffer (pH 9.6). Then each plate was rinsed three times with a washing buffer (PBS with 0.05% Tween 20). Blocking solution (5% BSA in washing buffer) was added to the ELISA plate at 80 µL per well for incubated at room temperature for 2 hours. Then the plate was rinsed three times with the washing buffer, and serum samples (diluted with washing buffer) were added in duplicate at 80 µL per well. The plate was incubated at 4 °C overnight. After washing with washing buffer, 80 µL of detecting secondary antibody was added and incubated at room temperature for 2 hours. Finally, 80 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (BD bioscience, NJ, USA) was added to each well. The reaction was incubated at 37 °C for 30 min and then stopped by adding 20 µL of 2.5 M H₂SO₄. The absorbance was read by an ELISA plate reader at 450 nm.

RESULTS AND DISCUSSION

Preparation of Icarapin Variant

The deduced amino acid sequences of transcript icarapin represent a polypeptide of 222 amino acids (GenBank accession number JF346659). A signal peptide cleavage site was identified between positions 19 and 20 (Fig. 2). Sequence comparison between icarapin from Asian honey bee (*A. cerana*) and the reported icarapin of European honey bee (*A. mellifera*, GenBank accession number ABF21077) showed that the latter has an inserted isoleucine between positions 43 and 44 of Asian honey bee. Also, there were 6 substitutions at positions 40, 66, 188, 191, 193 and 197 (position refers to Asian honey bee).

The aim of this work is to find out the potential antigenic sites of icarapin. It has been shown that glycosylation can influence the immunogenicity of proteins [12]. The three dimensional structure of wild type icarapin without signal peptide was predicted using program 3Dpro. The predicted structure contained only the CA atoms. Then, antigenic determinant of icarapin was searched and the top ten predicted continuous B-cell epitopes involved R32-T37, I109-V114, N125-K131, N125-I132, T126-K131, T126-I132, T127-I132, D162-N167, P197-E203 and S198-E203 (positions refer to icarapin with signal peptide). The third criterion of the potential site was determined by the location of O-glycosylated sites, which were predicted using NetOGlyc 3.1 Server. Result showed that eight threonines (T172, 173, 183, 184, 187, 191, 194 and 196) and one serine (S198) were the most likely O-glycosylation sites. Based on these three prediction results, we found that S198 met all the criteria. The chosen site was further confirmed by masking it with PEG. Codon at S198 was changed to cysteine by mutagenic PCR primers. Wild type and S198C variant could be expressed in *E. coli* and partially purified by HisTrap FF column (Fig. 3A).

Coupling of PEG to Modified Icarapin

The attached PEG masks the protein from the host's immune system and prevents antibodies from binding. In this study, thiol-selective PEGylation was used, which is highly specific to sulfhydryl groups. Since native icarapin has no cysteine residue, PEG-maleimide (activated PEG) could only be coupled to the free sulfhydryl group of cysteine residue introduced by mutagenesis. As a result, the PEGylation is site specific. Before PEGylation, DTT was added to the variants to break any disulfide linkages between cysteine residues, and excess DTT was removed by dialysis to prevent the inhibition of PEGylation process. The PEGylated S198C was separated from the uncoupled protein and PEG by SDS-PAGE and the PEGylated S198C variant was recovered from the gel by electro-elution. Wild type and S198C were also purified by SDS-PAGE and electro-eluted (Fig. 3B).

Immunogenicity: IgG and IgE Responses

The immunogenicity of icarapin and its derivatives was tested in mice. The result showed that IgG level induced by S198C was almost the same as the wild type protein (Fig. 4A). In contrast, there was 50% less in IgG level induced by PEGylated S198C throughout the dilution range. Similar

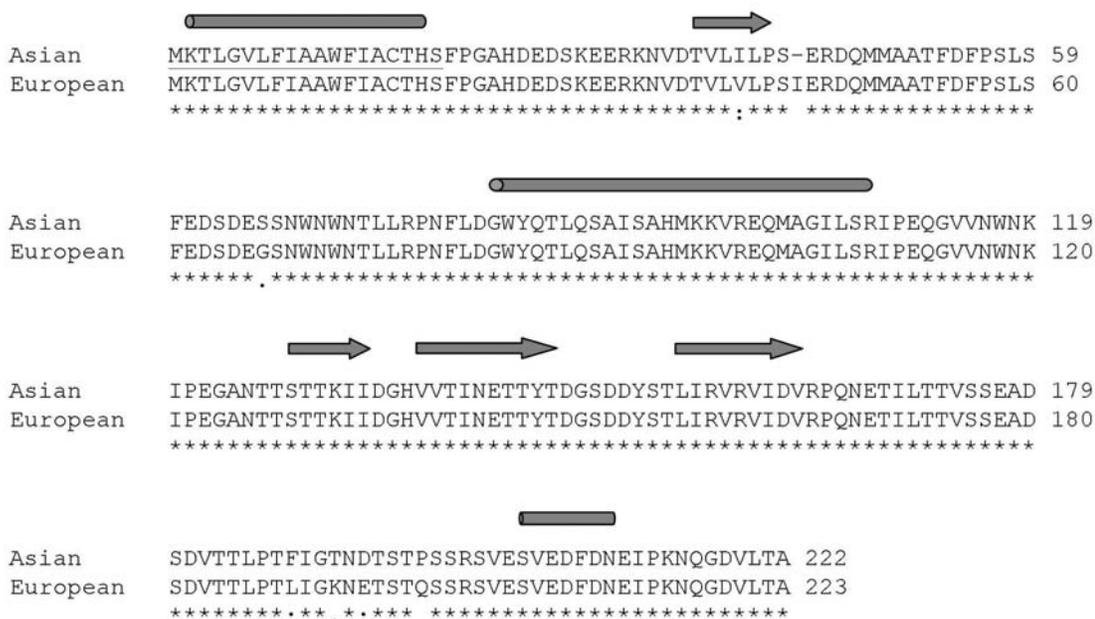


Figure 2. Protein sequence alignment between icarapin of Asian honey bee (Asian) and European honey bee (European). The premature icarapin of Asian honey bee contains 222 aa. The first 19 aa constitutes the signal peptide of icarapin, which is underlined. Asterisk denotes amino acid identical in two species. Single dot and double dot denote weakly and strongly similar amino acids, respectively. The cylinders and arrows show the predicted α helix and β strand of the icarapin of Asian honey bee, respectively.

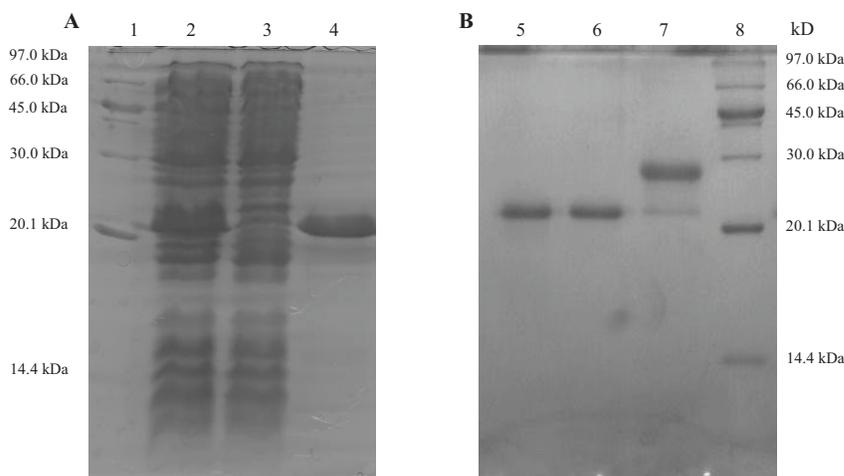


Figure 3. Purification of icarapin by (A) HisTrap FF column and (B) electro-elution as analyzed by 15% SDS-PAGE. (A) lane 1, low molecular weight marker (GE healthcare, WI, USA); lane 2, supernatant of crude bacterial lysate; lane 3, eluate from HisTrap FF column by buffer A; lane 4, S198C eluted by 300 mM imidazole in buffer A. (B) lane 5, wild type icarapin; lane 6, S198C variant; lane 7, PEGylated S198C variant; lane 8, low molecular weight marker.

observations were also found with the IgE response, though S198C elicited slightly less IgE than the wild type (Fig. 4B). As a result, S198C was confirmed to be an antigenic site of the icarapin.

PEG itself is a very weak immunogen. In this study, small PEG molecule ($M_r = 5000$) was used to mask the antigenic site. Besides PEG_{5k}, PEG_{20k} is also a common ligand

for reducing the antigenicity of proteins. However, smaller PEG is a better choice for mapping the location of the immunogenic site, and to mask several close by candidate antigenic sites on the protein [13].

In conclusion, we have identified an antigenic site in icarapin by in silico determination and confirmed its existence by site-specific PEG coupling. This approach may be

Fig. 4A.

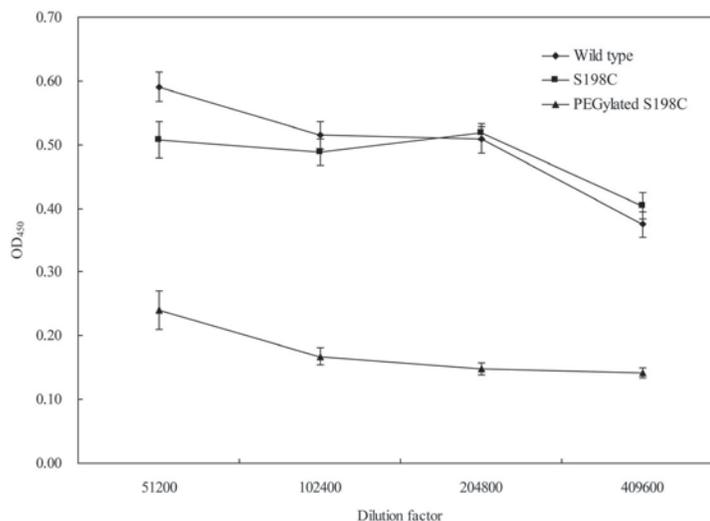


Fig. 4B

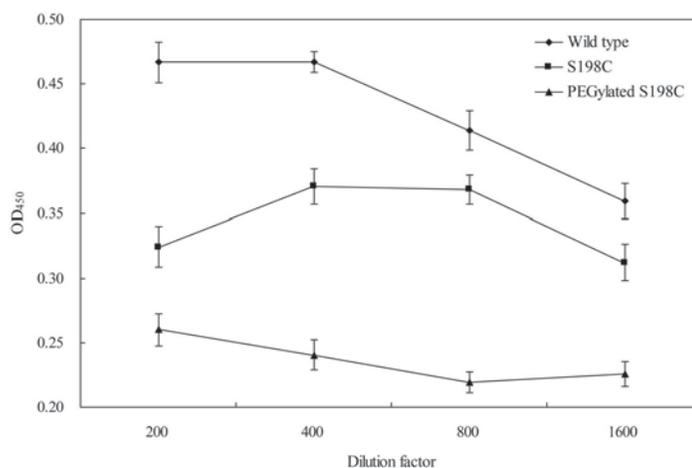


Figure 4. Icarapin specific (A) IgG and (B) IgE titre in mice immunized with wild type icarapin and its derivatives. A secondary antibody conjugated to horseradish peroxidase was used, with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. Values are means \pm SEM (N= 6). The plot uses log₁₀ scale for the x-axis.

extended to other antigenic proteins, for revealing and reducing their antigenicity.

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