

Y55 and D78 are crucial amino acid residues of a new IgE epitope on trichosanthin

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Abstract

Trichosanthin (TCS) possesses many biological and pharmaceutical activities, but its strong immunogenicity limits its clinical application. To reduce the immunogenicity of TCS, we modified the reported method for the prediction of antigenic site and identified two crucial amino acid residues (Y55 and D78) for a new epitope. We mutated these two residues into glycine and serine, respectively, and obtained three mutants, Y55G, D78S, and Y55G/D78S. These mutants induced less amount of Ig and IgG antibodies in C57BL/6J mice than wild-type TCS (wTCS) ($p < 0.01$) and almost lost the ability to induce IgE antibody production. The mutants stimulated fewer TCS-specific B cells in C57BL/6J mice than wTCS ($p < 0.01$). Compared with wTCS, Y55G, D78S, and Y55G/D78S lost 26.9%, 17.9%, and 98.7% specific binding ability to anti-TCS monoclonal antibody TCS4E9, respectively. These mutants still retained RNA N-glycosidase activity. In conclusion, Y55 and D78 are two crucial amino acid residues of a new IgE epitope on TCS, and their mutation reduces the immunogenicity of TCS, but still retained the enzymatic activity.

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Trichosanthin (TCS) is a plant protein isolated from the root tubers of a Chinese traditional herb *Trichosanthes kirilowii* Maxim. (Cucurbitaceae) [1]. This plant protein is a type I ribosome-inactivating protein (RIP) and consists of 247 amino acid residues [2] with a molecular weight of approximately 27 kDa. The three-dimensional structure of TCS is determined [3–5], and its putative active site is proposed [6,7]. TCS possesses a broad range of biological and pharmacological activities, such as anti-tumor [8,9], anti-viral [10], immunosuppressive [11], and mid-term abortifacient activities [12]. Clinically, TCS is used to induce mid-term abortion, treat ectopic pregnancies and hydatidiform moles, reset menstruation, and expel retained placenta. More important, it has ever been applied in the treatment of patients with AIDS or AIDS-related complex [10,13–16]. However, TCS is a strong allergen and can

induce type I hypersensitive reaction, and the TCS-specific IgE-secreting cells have been detected even three years after the termination of treatment [17]. Furthermore, specific antibodies can neutralize some TCS and speed up its plasma clearance, which makes frequent administration required to maintain an effective therapeutic concentration in the blood. These properties have greatly limited TCS as a therapeutic agent. Therefore, it is of great importance to reduce the immunogenicity of TCS.

Numerous studies have been carried out on the mapping of epitopes on the TCS molecule and developed many methods to reduce its immunogenicity. Gu et al. [18] investigated the epitopes by antibody competitive binding assay and classified 16 anti-TCS monoclonal antibodies (mAbs) into 4 groups, implying that there exist at least 4 epitopes on this molecule. By deleting the last 7 amino acid residues (LNRNNMA) from the C-terminus, Chan et al. [19] obtained a mini-TCS that shows a 2.7-fold decrease in antigenicity and a 10-fold decrease in ribosome-inactivating activity. Zhu et al. [20] predicted the antigenic epitopes

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by screening phage displayed random peptide library using mAb as a probe, and further confirmed the predicted sequence of 4B5-corresponding epitope by site-directed mutation. The 4B5- and 2E9-corresponding epitopes are located at amino acid residues 27–37(NERKLYDIPLL) and 41–48(SLPESQRY) of TCS protein, respectively. Cai et al. [21] adopted the similar method to locate TE1-corresponding epitope at amino acid residues 169–174(QQIGKR). Chan and He [22–24] identified the potential antigenic sites of TCS by computer modeling, and obtained four TCS mutants, S7C, K173C, Q219C, and K173C-Q219C by site-specific mutation; when coupled to PEG or dextran, these mutants show lower immunogenicity and longer plasma half-life. The above-mentioned antigenic sites are summarized and illustrated in Fig. 1A.

In this report, we analyzed the reported antigenic sites and three-dimensional structure of TCS, then modified the method described by Chan et al. [22], and identified two amino acid residues crucial for a new IgE epitope on the TCS molecule successfully. We obtained three mutants

by site-directed mutation, and verified that these mutants possessed lower immunogenicity than natural and wild-type TCS (nTCS and wTCS) and retained the RNA N-glycosidase activity of TCS.

Materials and methods

Materials. Alum adjuvant (Imject® Alum) was purchased from Pierce Chemical Company (USA). Fetal bovine serum (FBS), high-glucose DMEM, and HT supplement were products of Invitrogen Corporation (USA). Peroxidase-conjugated rabbit anti-mouse immunoglobulins were obtained from DAKO A/S (Denmark). Ninety-six-well microplates for cell culture or EIA/RIA were bought from Corning Incorporated (USA). CM Sepharose Fast Flow was from Amersham Biosciences Inc. (Sweden). Balb/c and C57BL/6J mice and Wistar rats were provided by Institute of Laboratory Animals, Chinese Academy of Medical Sciences, PR China.

The lectin-free ConA conditioned medium was prepared according to the method described by Spiess et al. [25]. Rabbit anti-nTCS polyclonal antibodies and mouse anti-TCS mAb (TCS4E9) were made in our laboratory by the conventional method. They were purified through affinity chromatography using a prepacked column Hitrap™ rProtein A FF (Amersham Biosciences Inc.). The liver ribosomes were prepared from male Wistar rats according to the method by Spedding [26].

Selection and mutation of antigenic sites. We adopted the method described by Chan et al. [22] with slight modifications to select the potential antigenic sites of TCS. In brief, with reference to the three-dimensional structure of TCS [4,5] and previous reports on the antigenic sites [18–24], we considered four parameters of a particular region: (1) exposure on the surface, (2) formation of loops or turns, (3) large size or strong polarity of amino acid side chains, and (4) remoteness from the active site. Two sites, Y55 and D78, were selected, which showed highest probability to induce antibody response and were away from the known antigenic sites and active site as illustrated in Fig. 1.

wTCS gene was cloned from the genome of *T. kirilowii* by PCR (GenBank Accession No. AY669811), and the mutant genes were constructed by recombinant PCR. The PCR-generated DNA fragments were sequenced to ensure that no secondary mutation had occurred in the sequence. All these genes were cloned into a prokaryotic expression vector pT7-7 with *NdeI* and *HindIII* sites.

Preparation of TCS and its mutants. Expression and purification of wTCS and its mutants were performed as previously described by Mi et al. [27]. In brief, the wTCS and its mutants were expressed in *Escherichia coli* strain BL21(DE3), and the proteins were purified by ammonium sulfate fractional precipitation and subsequent chromatographic separation on the CM Sepharose fast flow column. Natural TCS (nTCS) was purified following the same protocol except pulverizing the tuber roots of *T. kirilowii* instead of lysing the *E. coli*. Purified proteins were examined by 12% SDS-PAGE and Western blotting and quantified by the bicinchoninic acid (BCA) assay [28].

Immunization. Male C57BL/6J inbred mice (6–8 weeks old) were used. Thirty mice fell into 6 groups randomly, and each group consisted of 5 mice. The mice were immunized by i.p. injection with 10 µg/0.2 ml TCS emulsified in alum adjuvant on day 0. Two booster immunizations were given on day 14 and 28, respectively. Blood samples were collected on day 35 for the detection of TCS-specific antibodies. Spleen cells were isolated and used to detect the secretion of specific antibodies and count the TCS-specific B cells. Each experiment was repeated three times.

Indirect ELISA for the detection of TCS-specific antibodies. TCS-specific Ig, IgG, and IgE antibodies were detected by indirect ELISA. EIA/RIA microplates were coated overnight at 4 °C with 100 µl of antigen (10 µg/ml) in coating buffer (0.05 mol/L pH 9.6 NaHCO₃–Na₂CO₃), rinsed with PBST (0.01 mol/L PBS containing 0.05% Tween 20), and then blocked with blocking buffer (1% BSA in PBS) at 37 °C for 2 h. Serum samples diluted in PBST were added in duplicate to EIA/RIA microplates at 100 µl/well. After incubation and washing, 100 µl of detecting secondary antibodies (peroxidase-conjugated anti-mouse Ig, IgG, and IgE for measuring Ig, IgG, and IgE, respectively) was added to each well. Finally,

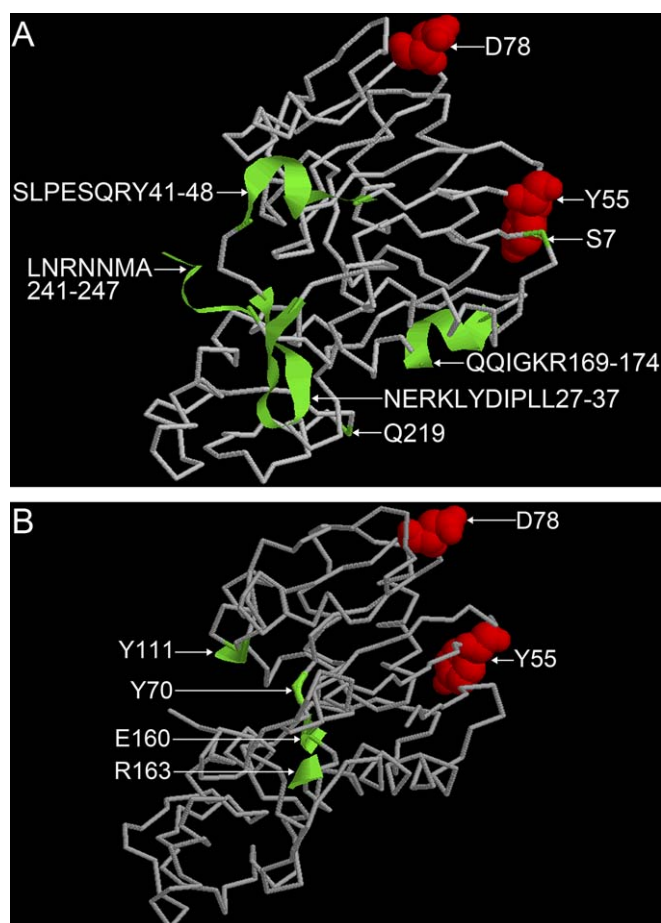


Fig. 1. Y55 and D78 were selected as candidate amino acid residues crucial for an epitope on the TCS molecule. These two sites were (A) located at the region different from the known ones (shown in ribbons model) and (B) far from the active site (the crucial residues were shown in ribbons model). In this study, Y55 and D78 were mutated from tyrosine and aspartic acid to glycine and serine, respectively. The structures were generated by software RasMol v2.7.

100 μ l of TMB solution was added to each well and developed color at darkness for 15 min. The reaction was stopped by adding 50 μ l/well 2 mol/L H_2SO_4 . The OD values were read on an EL311 automatic microplate reader (Bio-Tek Instruments Inc., USA) at 450 nm. For measuring Ig and IgG, serum samples were diluted 1:5000, whereas for IgE measurement, serum samples were detected at a dilution of 1:100.

Assay for the secretion of cultured spleen B cells. The spleen cells were aseptically isolated from the immunized mice and washed three times with PBS. The cell pellet was suspended to a concentration of 1×10^7 /ml in IMDM containing HT (1 $\times$), 0.5% ConA conditioned medium, and 5% fetal bovine serum. Two hundred microliters of the cell suspension was added to a 96-well culture microplate and cultured at 37 °C in 5% CO_2 atmosphere for 16 h. The culture supernatants were detected for the determination of TCS-specific Ig, IgE, and IgG by the indirect ELISA as described above.

Enzyme-linked immunospot assay for counting TCS-specific B cells. The spleen cells were prepared as described above. Ninety-six-well enzyme-linked immunospot (ELISPOT) microplates were coated overnight at 4 °C with 50 μ l/well TCS solution (20 μ g/ml in coating buffer), rinsed three times with PBS, and then incubated with 100 μ l/well 1% BSA at 37 °C for 1 h. After washing with PBS, 100 μ l of spleen cell suspension (2×10^7 /ml) was added to each well and incubated at 37 °C in 5% CO_2 atmosphere for 15 h. After washing with PBST, peroxidase-conjugated secondary antibodies were added to each well and incubated at 37 °C for 3 h. 3-Amino-9-ethylcarbazole (AEC) solution was added to develop color in the darkness at room temperature. The plates were rinsed five times with deionized water and dried at room temperature. The colored spots were counted under a stereomicroscope at a 25-fold magnification.

Assay for the binding ability of TCS mutants with TCS4E9. The relative specific binding ability of TCS mutants to TCS4E9 was determined by the indirect ELISA as described above except that the primary antibody was TCS4E9 instead of serum samples. Mouse anti-TCS polyclonal antibodies were used to serve as positive control.

Assay for RNA N-glycosidase activity. The assay for RNA N-glycosidase activity of TCS was performed as described by Endo and Tsurugi [29] with modifications. In brief, the ribosomes were incubated with 10 ng TCS in 100 μ l reaction mixture at 37 °C for 15 min. After phenol:chloroform extraction and ethanol precipitation, the resulting rRNAs were treated with acidic aniline at 50 °C for 10 min, and the released specific fragments (R-fragment) were then fractionated on a 5% (w/v) polyacrylamide gel containing 8 mol/L urea and stained with ethidium bromide.

Results

Mutation, expression, and purification of TCS and its mutants

Y55 and D78 were selected as potential antigenic sites (Fig. 1). We mutated Y55 from tyrosine to glycine and D78 from aspartic acid to serine by recombinant polymerase chain reaction (PCR) method, and obtained three mutants, Y55G, D78S, and Y55G/D78S. Sequence analysis verified that there was no secondary mutation occurring in the PCR-generated DNA fragments. The genes were cloned into a prokaryotic expression vector pT7-7 and expressed at a high level (~ 24 mg/L) in *E. coli* strain BL21(DE3). The proteins were purified by ammonium sulfate fractional precipitation and subsequent chromatographic separation on a CM Sepharose fast flow column (Amersham Biosciences Inc., Sweden). As shown in Fig. 2, the purified proteins presented a single band on a 12% SDS–polyacrylamide gel and possessed strong specific

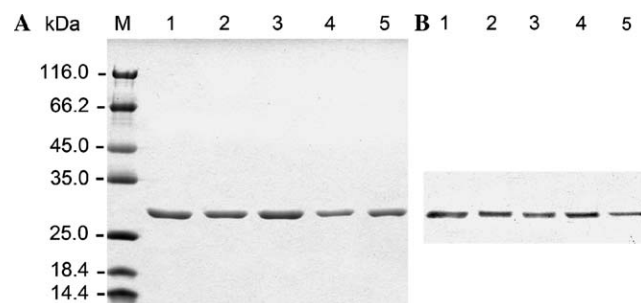


Fig. 2. Analysis of purified TCS and its mutants by SDS–PAGE and Western blotting. (A) After purified by ammonium sulfate fractional precipitation and subsequent chromatographic separation through CM Sepharose, all the purified proteins showed a single band on the 12% SDS–PAGE gel. (B) The purified proteins were further confirmed by Western blotting analysis. After running on a 12% SDS–PAGE gel, the proteins were transferred onto a nitrocellulose membrane. Then, they were detected with rabbit anti-nTCS polyclonal antibodies and subsequently HRP-conjugated goat anti-rabbit IgG. The membrane developed color with AEC solution at darkness. M, protein markers; lane 1, nTCS extracted from *T. kirilowii*; lane 2, wTCS; lane 3, Y55G; lane 4, D78S; lane 5, Y55G/D78S. The latter four proteins were expressed in *E. coli* strain BL21(DE3).

immunoreactivity to rabbit anti-nTCS IgG as analyzed by Western blotting.

Immunogenicity of TCS and its mutants

To evaluate the immunogenicity of these TCS mutants, we immunized male C57BL/6J mice with nTCS, wTCS, Y55G, D78S, and Y55G/D78S, respectively, and PBS was used as negative control.

The serum Ig, IgG, and IgE antibody levels were determined by ELISA and the results are shown in Fig. 3A. nTCS and wTCS exhibited similar ability to induce the production of Ig and IgG in C57BL/6J mice, whereas wTCS induced less amount of specific IgE than nTCS ($p < 0.01$). Compared with wTCS, the three mutants induced less amount of specific Ig and IgG antibodies ($p < 0.01$). More important, these mutants almost lost the ability to induce the production of IgE.

The spleen cells isolated from immunized C57BL/6J mice were cultured and the supernatants were used undilutedly to detect the relative levels of specific antibodies. Fig. 3B shows that the supernatants in nTCS and wTCS groups had similar antibody levels. Compared with wTCS group, Y55G and D78S groups had lower levels of specific Ig and IgG antibodies ($p < 0.05$), and Y55G/D78S group had much lower levels of specific antibodies ($p < 0.01$). Fig. 3B also indicated that there were fewer specific IgE antibodies in three mutant groups than in nTCS and wTCS groups ($p < 0.01$), and the figure also shows that their values were very low.

ELISPOT assay was used to count the TCS-specific spleen B cells isolated from TCS-immunized C57BL/6J mice. The nTCS- and wTCS-immunized mice exhibited similar levels of TCS-specific B cell counts, but the mutant-immunized mice had much fewer specific B cell counts ($p < 0.01$) (Fig. 3C).

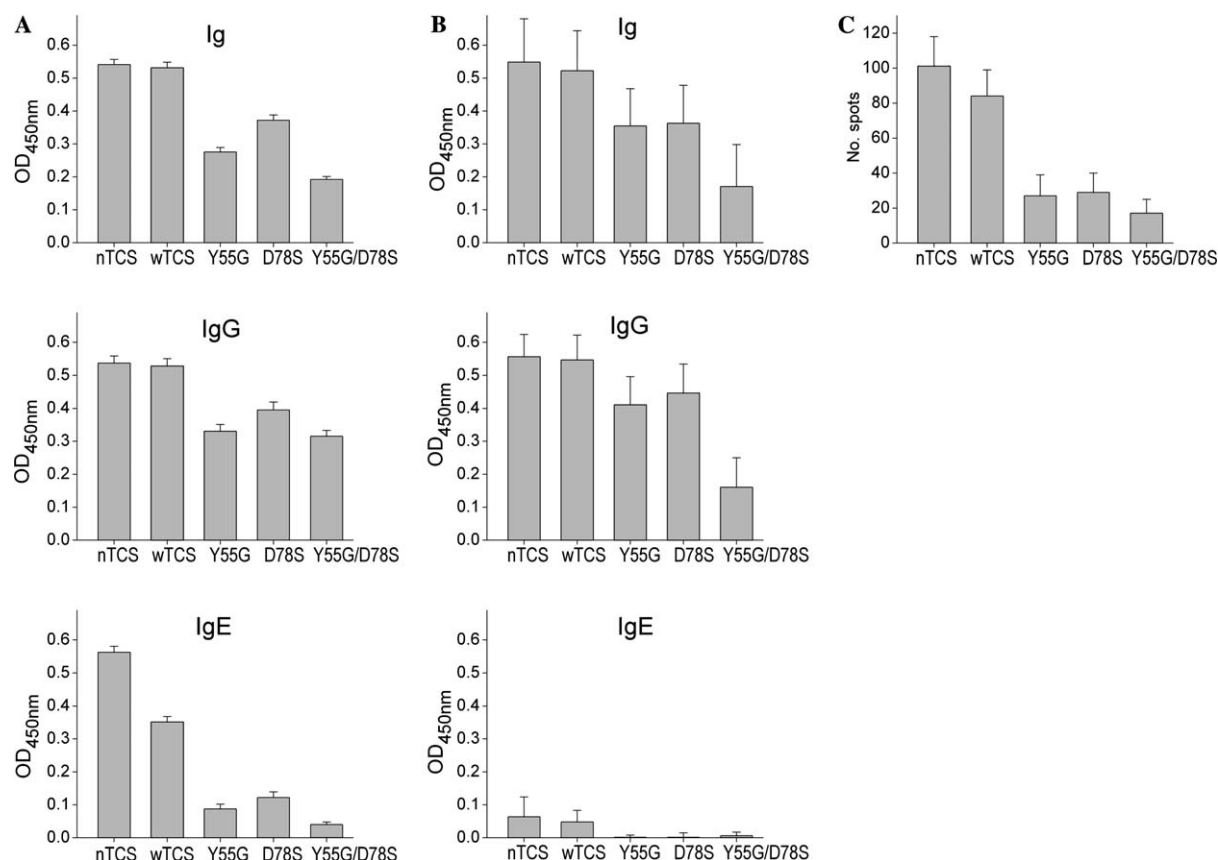


Fig. 3. Detection of the immunogenicity of TCS mutants. C57BL/6J mice were immunized with nTCS, Y55G, D55G/D78S, and sera and spleen cells were isolated for detection. Ninety-six-well EIA/RIA or ELISPOT microplates were coated with nTCS, wTCS, Y55G, D78S, and incubated with (A) serum samples to detect the relative amount of Ig, IgG, and IgE in the serum, (B) culture supernatants of spleen B cells to detect the relative amount of Ig, IgG, and IgE secreted by the cultured cells, and (C) spleen B cells to count the number of TCS-specific B cells in the spleen. Compared with nTCS and wTCS, all of the three TCS mutants almost lost the ability to induce IgE production, even though they still retained partial ability to produce TCS-specific Ig and IgG antibodies. There were fewer TCS-specific B cells in the spleens of C57BL/6J mice immunized with TCS mutants than with nTCS and wTCS.

Binding ability of TCS mutants to anti-TCS mAb

To determine whether Y55 and D78 belonged to the same epitope, we measured the relative specific binding activity of nTCS, wTCS, and three mutants to anti-TCS mAb TCS4E9 by indirect ELISA. Fig. 4 shows that nTCS and wTCS possessed similar ability to bind to TCS4E9. Compared with wTCS, the specific binding activity of Y55G, D78S, and Y55G/D78S to TCS4E9 was reduced by 26.9%, 17.9%, and 98.7%, respectively.

Biological activities of TCS mutants

To evaluate the effect of mutation on the biological activities, we determined one of the main activities of TCS, RNA N-glycosidase activity. Fig. 5 indicates that nTCS, wTCS, and three mutants could all make rat ribosomes release a ~450 nt specific fragment (R-fragment).

Discussion

TCS has a broad spectrum of biological and pharmaceutical activities, and is a potential therapeutic agent for

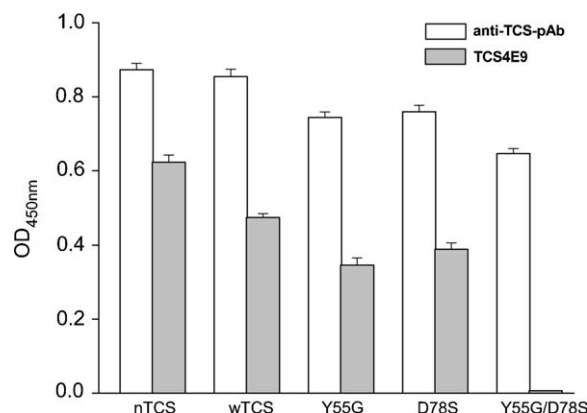


Fig. 4. Binding ability of TCS mutants with TCS4E9. All TCS mutants (Y55G, D78S, and Y55G/D78S) retained the ability to bind with anti-TCS polyclonal antibodies (anti-TCS-pAb), but Y55G and D78S retained only partial binding ability to anti-TCS mAb (TCS4E9), whereas, Y55G/D78S lost the specific binding ability to TCS4E9. Anti-TCS-pAb was mouse anti-TCS polyclonal antibodies.

multiple diseases, such as tumor and AIDS [8–10,13–16]. However, its strong immunogenicity limits its clinical applications. To reduce the immunogenicity of TCS,

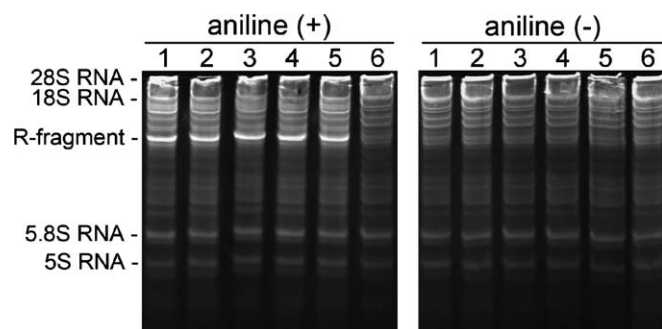


Fig. 5. RNA N-glycosidase activity of purified TCS and its mutants. Ribosomes isolated from the liver of Wistar rats were incubated with 10 ng TCS. After phenol:chloroform extraction and ethanol precipitation, rRNAs were treated with acidic aniline. The resulting rRNAs were separated on a 5% (w/v) urea–polyacrylamide gel, and stained with ethidium bromide. nTCS, wTCS, Y55G, D78S, and Y55G/D78S all exhibited RNA N-glycosidase activity, with a specific R-fragment shown on the gel. Lane 1, wTCS; lane 2, Y55G; lane 3, D78S; lane 4, Y55G/D78S; lane 5, nTCS as positive control; lane 6, water as negative control.

researchers have developed many methods, mainly including deletion [19], site-directed mutation [20], and site-directed modification by coupling TCS to polyethylene glycol [23,24] and dextran [22]. These methods all require that the exact location of an epitope be identified. Therefore, many methods were established to determine the amino acid residues crucial for an epitope. Currently, these methods mainly include screening phage displayed random peptide library using mAb as a probe [20,21] and computer modeling [22–24].

The known antigenic sites are summarized and illustrated in Fig. 1A. The figure shows that these antigenic sites are mainly distributed over the linking region formed by both small and large domains, but rarely over the other regions of the large domain. In addition, the putative active site is also located at the linking region (Fig. 1B). Fig. 1A also indicates that there is a cleft in the large domain which is far away from the linking region (active site). Based on these analyses, we inferred that there probably existed a new epitope distributed over the cleft region of the large domain. In order to find the crucial amino acid residues, we modified the computer modeling method by Chan et al. [22], and found that Y55 and D78 possessed the highest probability to induce antibody response.

Next, we inferred that if Y55 was mutated from tyrosine to glycine and D78 from aspartic acid to serine, respectively, the mutants would not lose the RNA N-glycosidase activity of TCS. That is because these two residues were far from the active site and such mutation would not greatly change the stereochemical structure of TCS. The experimental results indicated that the mutants retained the enzymatic activity of TCS (Fig. 5), which confirmed the above inference on the enzymatic activity of TCS mutants.

To determine whether such mutation reduced the immunogenicity of TCS, we immunized C57BL/6J mice with TCS and its mutants, detected the levels of serum antibodies

(Ig, IgG, and IgE) and the ability of spleen B cells to secrete antibodies (Ig, IgG, and IgE), and counted the TCS-specific spleen B cells. The results implied that such mutation did reduce the immunogenicity of TCS and both Y55 and D78 were crucial amino acid residues of a strong IgE epitope on the TCS molecule, which simultaneously induced the production of other classes of antibodies. First, three TCS mutants all induced lower levels of serum Ig and IgG antibodies in C57BL/6J mice than nTCS and wTCS, especially a much lower level of IgE antibody. Second, these mutants could all induce fewer specific B cells in C57BL/6J mice than nTCS and wTCS. When cultured *in vitro*, the spleen cells from mutant-immunized mice secreted less amount of specific Ig, IgG, and IgE antibodies than those from nTCS- and wTCS-immunized mice. The very low values for IgE detection in the study implied that there were very few IgE-secreting B cells in the spleen, which may be why Gu et al. did not obtain anti-TCS IgE hybridomas from the fusions of NS1 myeloma cells with spleen cells [30]. Third, Y55G and D78S retained part of the specific binding ability to anti-TCS mAb, whereas Y55G/D78S almost lost the ability to bind with the mAb completely. Fourth, as shown in Fig. 1, both Y55 and D78 are sterically located on both sides of the cleft, respectively, which makes it possible that these two amino acid residues belong to the same epitope.

In conclusion, we have modified the reported computer modeling method, proposed four parameters for the prediction of antigenic sites, and successfully identified two crucial amino acid residues, Y55 and D78, for a new IgE epitope on the TCS molecule. We have obtained three TCS mutants Y55G, D78S, and Y55G/D78S by site-directed mutation. These mutated TCS proteins have a lower immunogenicity (allergenicity) than nTCS and wTCS, but still retain RNA N-glycosidase activity. Our study confirms once again that it is feasible to predict the antigenic sites for an immunogen (allergen) by computer modeling and reduce its immunogenicity (allergenicity) by site-directed mutation.

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