ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2018; 17(2):158-170.

The Effect of Differentially Designed Fusion Proteins to Elicit Efficient Anti-human Thyroid Stimulating Hormone Immune Responses

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Received: 16 April 2017; Received in revised form: 4 July 2017 Accepted: 2 August 2017

ABSTRACT

The production of human thyroid stimulating hormone (hTSH) immunoassays requires specific antibodies against hTSH which is a cumbersome process. Therefore, producing specific polyclonal antibodies against engineered recombinant fusion hTSH antigens would be of great significance.

The best immunogenic region of the hTSH was selected based on in silico analyses and equipped with two different fusions. Standard methods were used for protein expression, purification, verification, structural evaluation, and immunizations of the white New Zealand rabbits. Ultimately, immunized serums were used for antibody titration, purification and characterization (specificity, sensitivity and cross reactivity).

The desired antigens were successfully designed, sub-cloned, expressed, confirmed and used for in vivo immunization. Structural analyses indicated that only the bigger antigen has showed changed 2 dimensional (2D) and 3D structural properties in comparison to the smaller antigen. The raised polyclonal antibodies were capable of specific and sensitive hTSH detection, while the cross reactivity with the other members of the glycoprotein hormone family was minimum and negligible.

The fusion which was solely composed of the tetanus toxin epitopes led to better protein folding and was capable of immunizing the host animals resulting into high titer antibody. Therefore, the minimal fusion sequences seem to be more effective in eliciting specific antibody responses.

Keywords: Cross reactivity; Fusion protein immunization; Immunoassay

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Measuring the human thyroid stimulating hormone (hTSH) or Thyrotropin levels as the first frontier line in hyperthyroidism and hypothyroidism diagnosis, remains to be under the spotlight.[1-3] Although immunoassays are the most common hTSH assessment platforms, anti-TSH antibody production in animal hosts is still an outstanding issue.[4,5] Since hTSH share high homology with other members of the glycoprotein hormone family like FSH, LH and HCG, the elicited polyclonal antibodies against whole hTSH protein lack the necessary specificity.[5,6] The members of the glycoprotein hormone family share an identical alpha chain and a highly homologous (40%) beta chain.[6] The C terminus region of the beta chain is usually the most heterologous region.[6,7] However, this region consists of about 30 amino acids which are too short to elicit good high titer antibody against the molecule in question. Given the high homology among these hormones, selecting an amenable region to elicit a specific antibody response is still a challenge.[6,7] On the other hands, a significant sequence homology which is reported to exist between the TSH sequences of human and animals, like mouse, rabbit and goat, exacerbates the problem of specific and high affinity monoclonal and polyclonal antibody production against hTSH.

Various biotechnological approaches have been implemented to circumvent the aforementioned problems. Recombinant protein design is one the appealing strategies to overcome the hurdles associated with specific and sensitive immunization of animals against h-TSH.[8,9] However, rational design of the final immunogen is the key to have a highly immunogenic recombinant antigen.[8,9] Bioinformatics approaches provide the necessary tools required to select the suitable regions for a recombinant protein,[10-12] design suitable drugs[13,14] and even understand their action mechanisms.[15-20] These approaches have become an inseparable part of the contemporary biology which leads to reduced number of required empirical studies.[10,21] Increased chances for unspecific responses and immune unresponsiveness due to high homology, are the logics behind the existing insist for inclusion of minimal sequences in recombinant protein design.[22,23] The designed proteins should be credible for high expression and immunogenicity,[24,25] while the resulting immune response should lead to specific and sensitive antibodies.[26] It should be taken into account that a carefully designed protein sequence is usually added to the recombinant proteins, as a fusion.[24] An optimum fusion protein could alleviate the induction of humoral immunity, compensate for low molecular weight of minimal antigens and rise the least amount of immunity.[24,27] The PII and P30 universal T cell epitopes from the C fragment of the tetanus toxin are among the commonly used fusion parts.[28,29] However, the inclusion of these epitopes may not increase the molecular weight properly. Therefore, studies like the one conducted by Chambers et al. have included an extra 46 residue region form the cartilage Oligomeric Matrix Protein (COMP) to satisfy the need for higher molecular weight and more solubility.[29]

Although the inclusion of a longer fusion protein could bring about better antigenic characteristics, unspecific immune responses against these sequences seem inevitable.[30,31] In the present study, we have tried to elicit a strong anti-hTSH antibody and assess the influence of COMP inclusion in protein expression, stability and its immunological consequences. Moreover, we aim to understand if the PII and P30 universal T cell epitopes could exert the expected immunological responses without additional fusion sequences. To this end, employing various in silico and in vitro methods we have designed two immonogenes containing a C terminus region from hTSH and two different fusion proteins. The first fusion consists of PII and P30 epitopes along with the COMP domain, while the second fusion consists of PII and P30 epitopes. The two antigens were produced in the prokaryotic hosts and used for the rabbit immunizations following the purification. Ultimately, the resulting immune responses were analyzed regarding their specificity, sensitivity and titration.

MATERIALS AND METHODS

Ethics Statement

This study was performed in strict accordance with animal use protocols during the entire experimental period. All procedures were performed according to the ethical guidelines of Faculty of Medical Sciences, Tarbiat Modares University based on the United States NIH Guide for the Care and Use of Laboratory Animals (publication no. 85–23). All performed each recombinant protein were measured in the far-UV range of 190-260nm for 2D structure analysis and in the near-UV range of 250-350 nm for 3D structure analysis. Circular

dichroism spectra were collected on a Jasco's J-810 spectrometer experimental protocols in this study were approved by the Ethical Committee of Faculty of Medical Sciences, Tarbiat Modares University.

Antigen Design and Gene Synthesis

The suitable TSH antigen was selected according to the method employed previously.[32] Although this region is highly conserve between the TSH sequences, low conservancy between the sequence of this region and the other glycoprotein hormones was the most important criteria to choose it. Low sequence similarity between these hormones would result in low cross reactivity. To design the first fusion, the PII and P30 tetanus epitopes and COMP were added to the N terminus of the selected pieces of C terminal region of β TSH (here after called as SR95-1). The second fusion protein also designed by the tetanus toxin epitopes of the first fusion added to the selected pieces of C terminal region of β TSH (here after called as SR95-2). Each part of the antigen was separated from the rest of the sequence by a G reach linker sequence. The 3D structures of the final antigens were predicted by threading method. I-TASSER server at:
http://zhanglab.ccmb.med.umich.edu/I-TASSER and the LOMETS servers at:
http://zhanglab.ccmb.med.umich.edu/LOMETS/ were used to predict the 3D structures of the final antigens. Moreover, the Rammpage and Prosa servers were used to assess the quality of the obtained results at:
http://mordred.bioc.cam.ac.uk/~rapper/rampage.php and https://swissmodel.expasy.org/qmean/.
The ProtParam server at:
http://web.expasy.org/protparam/ and SAPS tool at:
http://www.isrec.isb-sib.ch/software/SAPS_form.html were employed to predict the physicochemical features like molecular weight and stability of the designed protein. Theantigenicity predictions were performed by VaxiJen server at:
http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html. The surface accessibility of the final proteins was predicted by the NetSurfP at:
http://www.cbs.dtu.dk/services/NetSurfP/. The sequence of the designed protein was reverse transcribed to the DNA sequence using ExPASY translate tool at:
http://web.expasy.org/translate/.
The codon optimization of the final DNA sequences were performed by (according to the E. coli codon usage bias) Jcat tool at:
http://www.jcat.de/. Before the gene order from the Generay Biotech Co, Ltd. all the necessary alterations for the sub-cloning of the final genes into pET32a plasmid (adding BamH1 and EcoR1 restriction sites to 5'and 3' end of the gene and checking for correct expression frame) were done.

Protein Expression, Purification and Characterization

The designed genes were sub-cloned into pET32a plasmid using the same method employed in our previous study.[32] The gene expression (conventional IPTG induction), purification (conventional nickel column purification for inclusion bodies) and verification for both SR95-1 and SR95-2 were done adopting the methods employed by Mardsoltani et al.[32]

Circular Dichroism (CD) Spectroscopy and Intrinsic Fluorescence

The percentage of secondary structures for each recombinant protein was measured by dissolving 0.2 mg of each protein in phosphate buffered saline (PBS) (pH: 7.2). For each spectrum, 3 scans collected and smoothed and then corrected for the contribution of buffer. The intrinsic fluorescence method was used to analyze and confirm the 3D structures of the recombinant proteins. The Shimadzu spectrofluorometer model RF3100 was employed to record the intrinsic fluorescence of recombinant proteins. A solution of 0.5 mg protein in 1 ml PBS (*p*H: 7.2) was prepared for each protein. A 280 nm induction wavelength was employed and the emission wavelength of 300-450 nm was analyzed

In vivo Immunization

The purified recombinant fusion hTSH proteins (SR95-1 and SR95-2) were used for polyclonal antibody production in New Zealand white female rabbits (two rabbits for each fusion protein). The rabbits were first injected (intra-muscularly into the large muscle of the rear legs) with 500 µL of a stable emulsion consisted of PBS solution containing 500 µg of recombinant fusion hTSH protein and the same volume of Freund's complete adjuvant. One month later the rabbits were injected for the second time. The booster injections were given at 15 day intervals every other week with 250µg of recombinant fusion proteins mixed with incomplete Freund's adjuvant.

Immediately after the third injection, the first bleeding was done and repeated for two more times with 15 days interval. Protein A affinity chromatography column was used (according to the manufacturer's instructions) to purify the polyclonal antibodies (IgGs) from the rabbit serums. The protein A binding step was performed in 0.02M sodium phosphate, pH7.0, while the elution step was in 0.1M citric acid, pH3.0. The bound IgGs were eluted in four fractions and immediately neutralized to physiological pH by addition of 1M Tris-HCl buffer, pH9 and then concentrated to 1mg/ml using 50 kDa−molecular mass cutoff concentrators. A SDS page analysis was performed for the collected fractions (the sample buffer lack the 2 mercaptoethanol). An indirect ELISA method was employed to assess the rabbit serum antibody titers along with its cross reactivity against other members of the glycoprotein hormone family. To measure the antibody titer, the purified SR95-1 and SR95-2 proteins were diluted to 1µg/ml in 100 mM carbonate–bicarbonate coating buffer (28.6 mM Na2CO3, 71.4 mM NaHCO3, pH 9.6), and then100 µL/well were coated on a 96-well immunoplate at 4°Covernight. The wells were washed with PBS-T buffer (PBS containing 0.05% Tween 20) for three times and blocked with 200 µL of 5% skim milk for 2 h at 25°C, and then incubated 2 h at 25°C with 50 µL/well of serial diluted serum (1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000 in PBS) after washing the wells for three times. The wells were washed and incubated with 50 µL of 1:2000 diluted HRP-conjugated mouse anti-rabbit IgG (Thermo Scientific, MA, USA) for 1 h at 25°C. The wells were washed and 50 µL of tetramethyl benzidine substrate reagent (BD Biosciences Pharmingen, CA, USA) were added and incubated for 30 min at 25°C. Color development was stopped by the addition of 50 µL of 2.5 M of H2SO4 after 15 min, and the absorbance was measured at 450 nm. The cross-reactivity analysis was performed using the same procedure. 4 µIU of hTSH, LH, FHS and HCG along with 0.14 µgr of BSA were used to coat each wells, while 5 µgr of the purified antibody was added to each well to assess the cross reactivity according to the following formula:

Cross Reaction=(Optical Density (OD) of the Glycoprotein Hormone or BSA - OD of the PBS)/(OD of the hTSH–OD of the PBS) x 100

The sensitivity of assay designed for TSH detection by the produced antibody was assess by performing the ELISA test using 0.0, 0.625, 1.25, 2.5, 5 and 10 µg of the purified antibodies. 4 µIU of the TSH protein was coated on each well separately. Different concentrations of the purified antibodies against SR95-1 and SR95-2 proteins were added to assess the sensitivity of TSH detection by the antibodies produced in this study. Statistical significance of the obtained results were evaluated by one-way ANOVA using Statistical Analysis Systems Software (SAS) v9.4 (SAS Institute, Cary, NC, USA) followed by Duncan's multiple-range test.

RESULTS

Antigen Design and Prediction of Their Features

The protein sequence of the previously selected TSH region (spanning the amino acids 82-112)[32] along with two fusion moieties (first fusion is composed of the PII and P30 tetanus epitopes and COMP, while the second fusion is composed of the PII and P30 tetanus epitopes) was used to form the final antigens. Then, their corresponding genes were designed and optimized for prokaryotic expression (Figure 1).

The 3D structures of the final antigens along with their quality assessment results (Figure 2) indicated the structural properties of the final antigens. Since more than 90% of the amino acids resides within the allowed regions of the Ramachandran plots, the quality assessment of the predicted structures verifies the accuracy of the predictions. Moreover, the Prosa Zscores put these structures in the zone of previously resolved structures which indicates the good quality of the models. The predicted molecular weight for the SR95-1 protein was about 25 kDa and SR95-2 was about 35 kDa. Moreover, the SR95-1 protein was predicted as unstable protein, while SR95-2 was predicted as stable. The antigenicity scores were predicted to be 0.64 and 0.77 for SR95-1 and SR95-2 antigen, respectively. The respective surface accessibility for SR95-1 and SR95-2 antigens was predicted to be 58% and 68%.

Gene Sub-Cloning and Confirmation

The sub-cloning process was successfully carried out and the accuracy of the sub-cloning process was confirmed by the results obtained from the colony PCR and the sequencing of the plasmids purified from the achieved clones. The final sequences of each gene are depicted in Figure3.

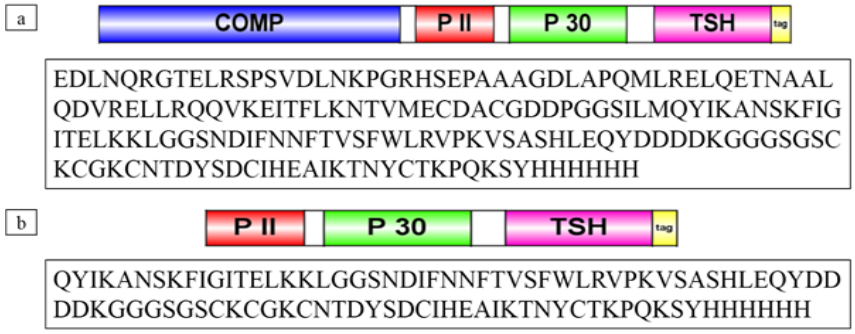

Figure 1. The sequences of the final proteins are named as SR95-1 (a) and SR95-2 (b) to assess the effect of differentially designed fusion proteins to elicit efficient anti-human thyroid stimulating hormone immune responses. The sequences of the designed immunization proteins along with their schematic illustrations are depicted. For both SR95-1 (a) and SR95-2 (b) schematic sequence illustrations: COMP stands for COMP fragment, PII and P30 stands for PII and P30 tetanus toxin epitopes, TSH is the hTSH sequence, the tag is the his tag sequence and the while linkers are the Glycine-rich linker sequences.

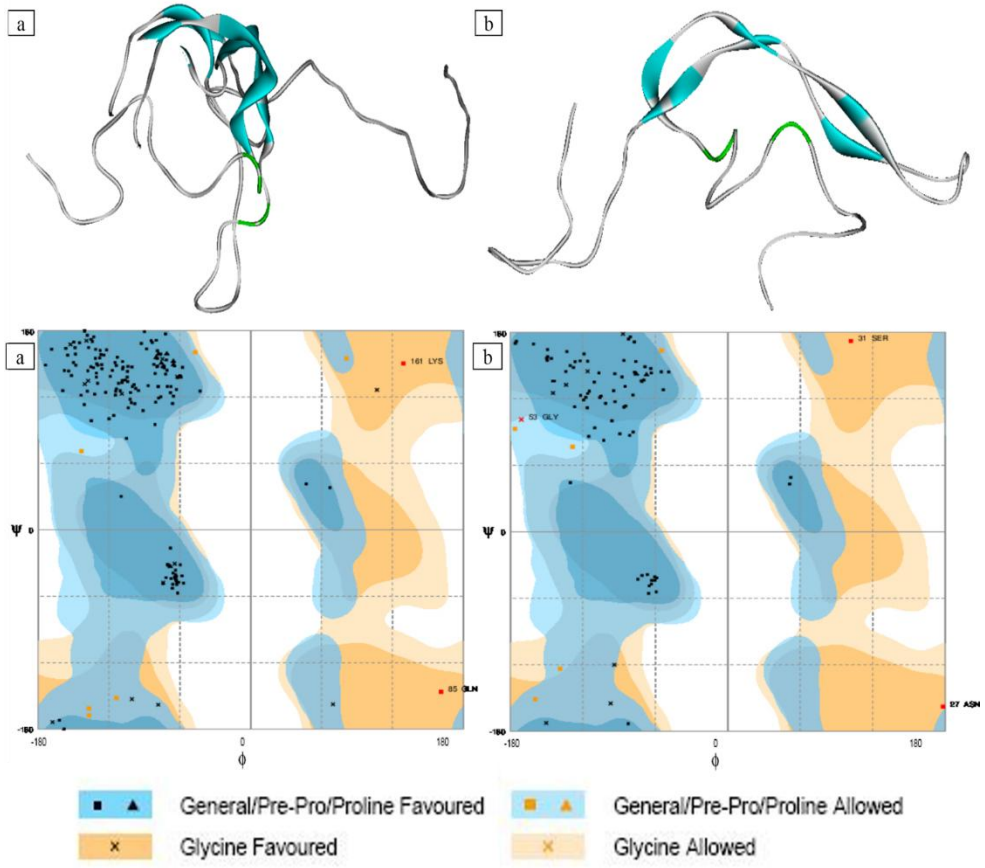

Figure 2.3D structure prediction and its quality assessment for SR95-1 and SR95-2 antigens to investigate the effect of differentially designed fusion proteins to elicit efficient anti-human thyroid stimulating hormone immune responses. (a) is the 3D structure of the SR95-1 antigen, (b) is the 3D structure of the SR95-2 antigen, (c) is the Ramachandran plot for the quality of the predicted SR95-1 structure and (d) is the Ramachandran plot for the quality of the predicted SR95-2 structure. The Ramachandran plots of the two fusion antigens indicate the accuracy of the predicted structures, due to the existence of more than 90% of the amino acids within the favored regions of the plot.

a:
GAAGATCTGAACCAGCGTGGTACCGAGCTGCGTTCTCCGTCTGTTGACCTGAACAAACCGGGTCGCC
ATTCTGAGCCGGCAGCGGCAGGTGACCTGGCTCCGCAGATGCTGCGTGAGCTGCAGGAAACTAACGC
GGCACTGCAGGATGTGCGCGAACTGCTGCGCCAGCAGGTTAAAGAAATCACCTTCCTGAAAAACACC
GTTATGGAATGCGACGCGTGCGGTGACGATCCGGGTGGTTCCATCCTGATGCAGTACATCAAAGCGAA
CTCTAAATTCATCGGTATCACCGAACTGAAGAAGCTGGGTGGCTCTAACGACATCTTCAACAACTTCA
CCGTTTCTTTCTGGCTGCGCGTGCCGAAAGTTAGCGCGTCTCACCTGGAACAGTACGATGACGACGAT
AAAGGTGGCGGCTCTGGTAGCTGCAAATGCGGCAAATGTAACACTGATTATTCTGACTGCATTCACGA
GGCTATCAAGACCAACTATTGTACCAAGCCGCAGAAGAGCTATCACCATCATCACCACCACtaa

b:
CAGTACATCAAAGCGAACTCTAAATTCATCGGTATCACCGAACTGAAGAAGCTGGGTGGCTCTAACGA
CATCTTCAACAACTTCACCGTTTCTTTCTGGCTGCGCGTGCCGAAAGTTAGCGCGTCTCACCTGGAAC
AGTACGATGACGACGATAAAGGTGGCGGCTCTGGTAGCTGCAAATGCGGCAAATGTAACACTGATTAT
TCTGACTGCATTCACGAGGCTATCAAGACCAACTATTGTACCAAGCCGCAGAAGAGCTATCACCATCAT
CACCACCACtaa

Figure 3. The DNA sequences of the final antigens after the codon optimization and sub-cloning confirmation to evaluate the effect of differentially designed fusion proteins to elicit efficient anti-human thyroid stimulating hormone immune responses. (a) is the DNA sequence for the first fusion protein (SR95-1) and (b) is the DNA sequence for the second fusion protein (SR95-2).

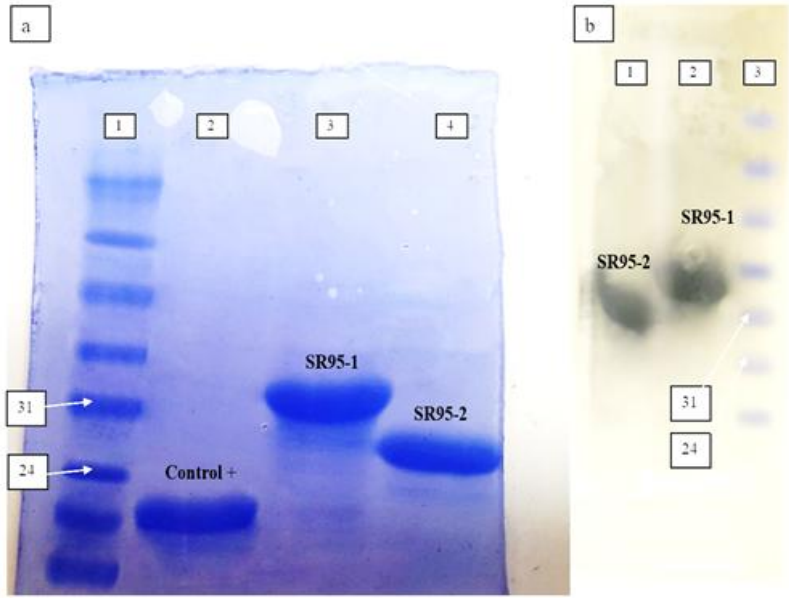

Figure 4. Protein expression analysis to assess the effect of differentially designed fusion proteins to elicit efficient anti-human thyroid stimulating hormone immune responses. (a) is the Sodium Dodecyl Sulfate (SDS) page results of the expressed fusion SR95-1 and SR95-2 antigens after purification (1 is the protein ladder, 2 is a nonrelated positive control, 3 is the first fusion protein and 4 is the second fusion protein). (b) is the Western blot analysis of the purified SR95-1 and SR95-2 antigens (1 is the SR95-2 protein, 2 is the SR95-1 protein and 3 is the protein ladder)

Protein Expression, Purification and Characterization

The best conditions for protein expression were determined to be 18°C with the 1mM concentration of the IPTG. Figure 4a depicted the protein expression results after the protein purification step which resulted in more than 80% purification. The Western blot confirmation of the purified proteins, using HRP-conjugated anti-his tag antibody, is shown in Figure 4b. Considering the 12 kDa extra protein sequences added by the pET32a plasmid, the total molecular weight of the expressed proteins for the first and second fusion proteins should be 32 and 22 kDa. The obtained bands in the expected molecular weight in the Western blot results confirm the successful expression and purification of the respective proteins.

2D and 3D Structure Analyses Results and Their Comparison with Bioinformatics

The results of CD for the first and second proteins along with their bioinformatics results are presented in Table 1. There is a more acceptable concordance between the results of empirical and average bioinformatics analyses for the SR95-2 protein. The results of 3D structure analyses for SR 95-1 and SR 95-2 recombinant protein are presented in Figure5.The results in Figure5 indicate that (the interpretation of results was based on study conducted by Micsonai et al 2015 and Nagatomo et al 2013) [33-36] the SR95-2 is folded properly, while the SR95-1 suffers from poor folding. The results of intrinsic fluorescence analyses are in line with the Near UV CD results and showing disordered 3D structure for the SR95-1 protein and proper 3D structure for the SR95-2. The emission intensity of the SR95-2 is 136 (336 nm wavelength), while the emission intensity of the SR95-1 is 78.5 (336 nm wavelength). The poor 2D and 3D structures of the SR95-1, in comparison to the SR95-2, could be rooted in its higher MW and lack of suitably formed secondary structures.

In vivo Immunization

Titration, sensitivity and cross reactivity analysis were performed using the second fractions from the purification step. Since even the 1:32000 dilutions of the collected serums are capable of SR95-1 and SR95-2 proteins detection (Figures 6a and 6b), the immunization regimen successfully elicited high titers of the respective antibodies for both fusion antigens. Moreover, the longer immunization duration is direct correlate with the higher antibody titer in blood. The sensitivity diagram (Figure 6c) apparently indicates that the antibody responses are getting stronger by increased concentrations of the obtained antibodies, while the response against BSA is constant. The minimal cross reactivity of the achieved antibody against the other members of the glycoprotein hormone family indicates the high specificity of the antibody (Table 2). As can be depicted from these results, the polyclonal antibody (SR95-2) exhibits almost no cross reaction with other members of glycoprotein family. The differences between antibody responses of SR95-1 and SR95-2 proteins were significant by p-value< 0.05.

Table 1. The results of protein 2D structure analyses using far ultra violet circular dichroism(UVCD) and bioinformatics tools to assess the effect of differentially designed fusion proteins to elicit efficient anti-human thyroid stimulating hormone immune responses

	Turn (%)	Random Coil (%)	Beta Sheet (%)	Alpha Helix (%)
SR95-1, 2D Structure Predicted by Jpred	9.5	14.89	30.95	44.66
SR95-1, 2D Structure Predicted by APSSP	0	48.5	11.5	40
SR95-1, 2D Structure Predicted by CFSSP	7/5	13	31/5	48
SR95-1, 2D Structure Predicted by GOR IV	0	55.3	17.2	27.5
SR95-1, 2D Structure Predicted by SOPMA	9	35	14	42
Average prediction	5.2	33.2	21	40.4
SR95-1, 2D Structure analysis by Far UV CD	26	35.9	26.6	11.5
SR95-2, 2D structure Predicted by Jpred	10.10	39.4	16.16	34.34
SR95-2, 2D structure Predicted by APSSP	0	42	19	39
SR95-2, 2D structure Predicted by CFSSP	10	6	35	49
SR95-2, 2D structure Predicted by GOR IV	0	63	18.5	18.5
SR95-2, 2D structure Predicted by SOPMA	10	37	17	36
Average prediction	10	37	21	35
SR95-2, 2D structure analysis by Far UV CD	20.3	35.1	11.4	33.2

The composition of protein secondary structures is predicted by Jpred, APSSP, CFSSP, GOR IV and SOPMA servers and the Far UV CD method. APSSP: Advanced protein secondary structure prediction, CFSSP: Chou-Fasman prediction of the secondary structure of proteins, GOR IV Secondary Structure Prediction Method, SOPMA: The self-optimized prediction method with alignment.

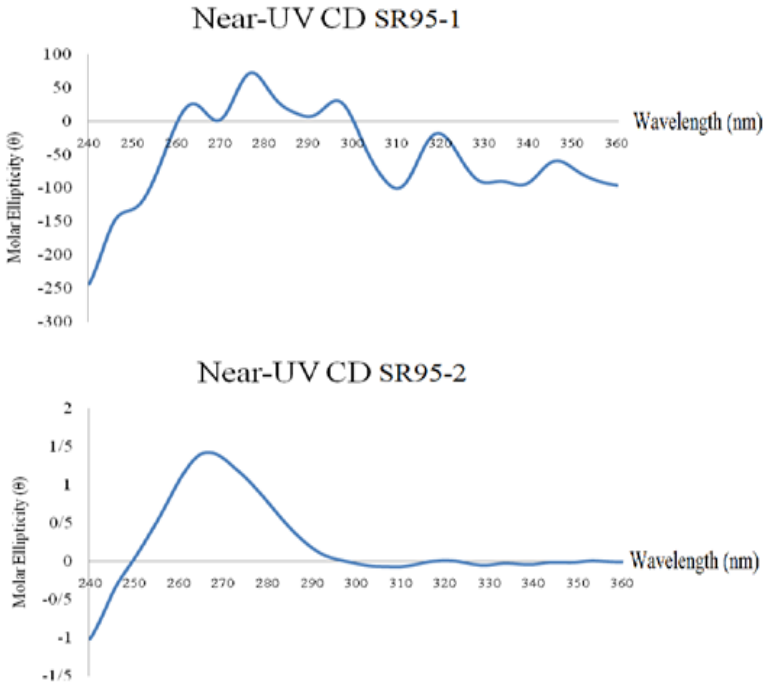

Figure 5. 3D structure analysis of SR95-1 and SR95-2 by Near UV CD. The upper plot is for the Near UV CD of the SR95-1 antigen and the lower one is for the SR95-2 antigen. Protein solutions were prepared dissolving 0.2 mg of each protein in phosphate-buffered saline (PBS) (pH: 7.2). Measurements were made soon after preparing the solutions.

Table 2. The cross reactivity analysis for the elicited and purified polyclonal antibodies after the in vivo immunization by both SR95-1 and SR95-2 fusion proteins. The cross reactivity percent is reported for LH, FSH and HCG.

Anti-First Fusion Protein (Anti-SR95-1) (50 µg/mL, 100 µL/well)	**Human thyroid-stimulating hormone (hTSH), Luteinizing hormone (LH), Follicle-stimulating hormone (FSH) and Human chorionic gonadotropin (HCG) Coated on microtiter plate (40 µIU/mL, 100 µL/well) or BSA Coated (4 µg/mL, 100 µL/well)**					
	Htsh	**LH**	**FSH**	**HCG**	**BSA**	**PBS**
Optical Density (O.D.) (450 nm)	1.681	0.459	0.482	0.469	0.402	0.388
% Cross Reaction	100%	5.49%	7.29%	6.26%	1.08%	**
Anti-Second Fusion Protein (Anti-SR95-2) (50 µg/mL, 100 µL/well)	**hTSH, LH, FSH and HCG Coated on microtiter plate (40 µIU/mL,100 µL/well) or Bovine Serum Albumin(BSA) Coated (4 µg/mL, 100 µL/well)**					
	Htsh	**LH**	**FSH**	**HCG**	**BSA**	**PBS**
O.D. (450 nm)	1.482	0.368	0.354	0.371	0.349	0.343
% Cross Reaction	100%	2.19%	0.9%	2.45%	0.5%	**

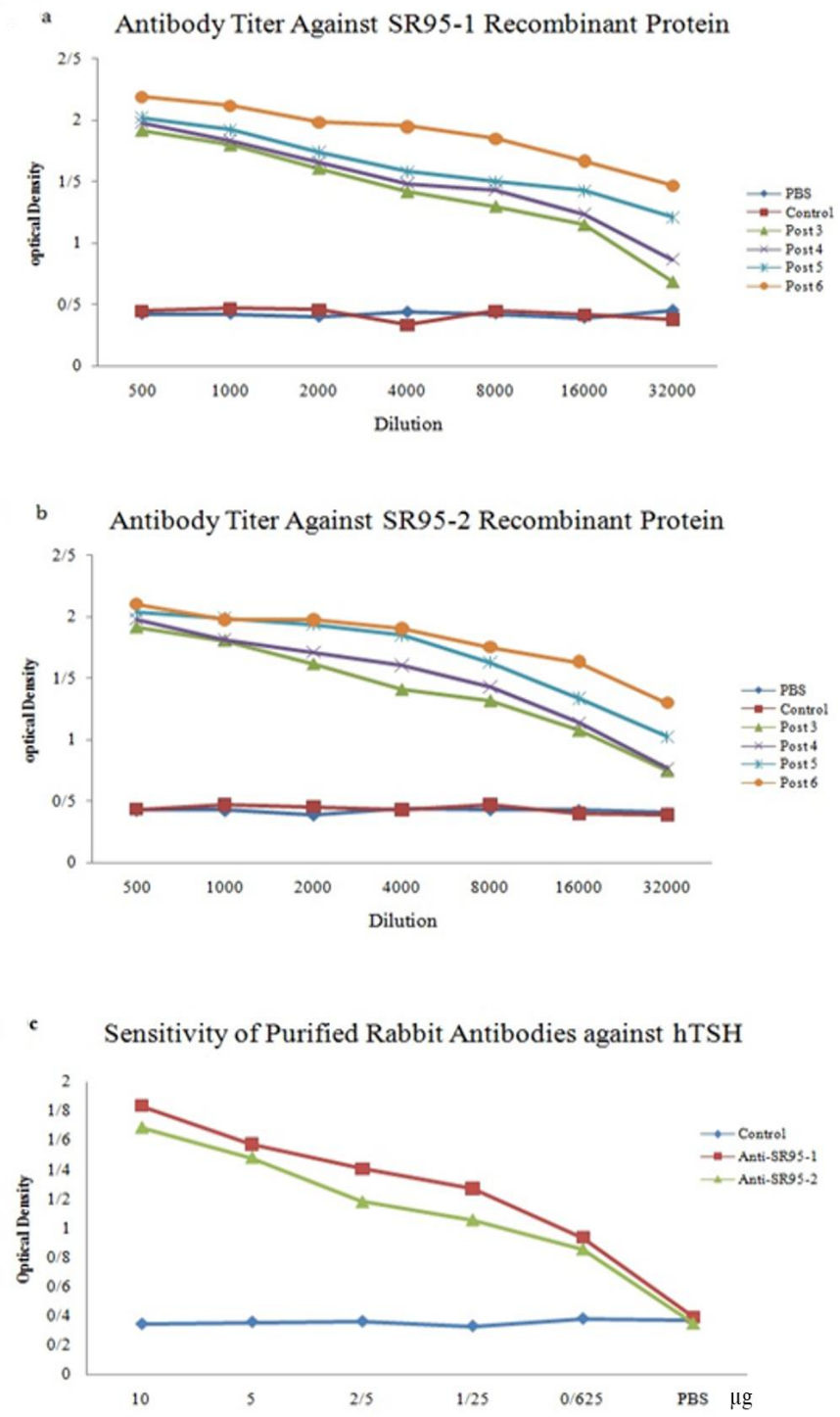

Figure 6. Antibody titration curve results. (a) Titration curve for the raised antibody against SR95-1 protein. (b) Titration curve for the raised antibody against SR95-2 protein. (c) Sensitivity curve for the purified rabbit antibodies against hTSH. (a) and (b) indicate that the antibodies obtained from the first (Post 3: after third injection), second (Post 4: after fourth injection), third (Post 5: after fifth injection) and fourth (Post 6: after sixth injection) bleedings, have high titers of the antibody, while the pre-bleed and PBS controls do not show any significant OD.

DISCUSSION

Production of a highly specific and sensitive antibody against the hTSH is an imperative requirement for hTSH diagnosis.[32] However, lack of an amenable immunogen has always been a tough challenge to raise strong humoral responses.[6,7,32] This issue is mainly rooted in the existing homology between the members of glycoprotein hormone family including TSH, LH, FSH and HCG. Over 90% sequence conservancy between the hTSH and the TSH sequence of common immunization hosts additionally contributes to worsening the problem. Thus, choosing the whole hTSH hormone or the beta chain to be administered as the immunogen would not be sufficient for polyclonal or monoclonal antibody production in host animals.[29,32] Although the whole hTSH hormone or the beta chain is endowed with the necessary molecular weight, the obtained responses would contain nonspecific antibodies. The regions of hTSH with low homology to other members of the glycoprotein hormone family could be the rational choice to circumvent aforementioned limitations.[22,30,31] The C terminal region of the members of this family β chain has been shown to be the region with highest variability. Our results have confirmed that the highest variability of these hormones belongs to the β hTSH C terminal region. This seems to be the logic behind the selection of this region as the immunogen in previous studies.[6,7] The highest antigenicity, high accessibility of epitopes and high number of linear B cell epitopes are the features associated with this region. Our results indicated that there is another variable region at the middle of the β hTSH sequence which could also be considered as a part of the designed immunogen. However, post translational modifications like disulfide bond and N-linked glycosylation at this region could hamper the linear epitope presentation process and consequently diminish its immunogenic properties.

Although the amino acids of the C terminus region harbors the necessary immunological properties, its utilization as the sole immonogene would be insufficient due to its low molecular weight (~3 kDA). It previously has been demonstrated that low molecular weight immunogens (<5 kDa), especially those with homology to the host homologs, could suffer from the low immunogenicity.[31] In the case of β hTSH C terminus region both low molecular weight and high identity to the host homolog were applicable. Therefore, two fusion proteins (SR95-1: COMP + PII and P30 Tetanus Toxin epitopes + Cut Pieces of C Terminus region of β TSH, SR95-2: PII and P30 Tetanus Toxin epitopes + Cut Pieces of C Terminus region of β TSH) were designed and added to the selected hTSH sequence to compensate for its low immunogenicity and compare their efficiency. PII and P30 Tetanus Toxin epitopes are capable of inducing the CD4+ T cells which are necessary to have strong humoral responses. A humoral response without suitable elicitation of helper T cell response would not last long. These epitopes are universally immunogenic recognized by all primed donors, irrespective of their MHC haplotypes.[37]Based on the performed in silico analysis, both of the designed fusion proteins have the necessary structural and immunological properties for efficient expression (in prokaryotic cells), purification and immunization in host animals. The obtained empirical results were in line with these findings. However, antibody titration results revealed that the SR95-1 was more immunogenic in comparison to the SR95-2 protein. This finding contradicts with the predicted epitope accessibility of the SR95-2 protein which was reported to be better than the SR95-1 protein. These results were confusing given the results of 2D and 3D structural analyses. These results indicated that the SR95-2 protein acquires a better folding in comparison to the SR95-1 protein, therefore its antigenic accessibility should be higher. This could be construed as the priority of the molecular weight over the epitope accessibility of the immonogenes.[38,39]Numerous previous investigations have also suggest the credibility of this idea.[29,32,38,39]Nevertheless, the obtained polyclonal antibodies for both fusion proteins were capable of hTSH detection with minimal cross reactivity against other glycoprotein hormones. These results indicated the very accuracy of the selected region for immunization purposes. The cross reactivity results of the raised polyclonal antibodies indicated that the SR95-2 protein produced a more specific immune response in comparison to the SR95-1 protein. These results are in concordance with the previous studies regarding the unspecific immune responses elicited by the fusion systems.[31,38,40-42] The longer the added fusion is designed, the more the nonspecific immunity is raised. It could be deduced that using minimal fusion sequences with suitable molecular weight, stability and immunological characteristics would bring about

desired immunological responses against challenging antigens like hTSH.

It could be concluded that the precisely selected cut pieces of C terminus region of β TSHis capable of efficient immunogenicity in the context of both of the designed fusion proteins even if the protein is folded poorly. It should be pinpointed thatthe smaller fusion protein (the construct without COMP) has led to slightly weaker expression, purification and immune responses. However, the obtained polyclonal antibodies showed sensitivity and specificity values equivalent to monoclonal antibodies or even better than them. In this regard, it should be pointed out that commercially available monoclonal antibodies are of lower specificity (68.6%) against TSH (like TSH51) in comparison to their higher specificity (with 0.03%, 2.99% and 0.66% cross reactivity against hCG, hLH and hFSH respectively).[41] Inclusion of the COMP sequence in the SR95-1 protein has intriguingly led to better expression, purification and immunizations. On the other hands, the escalated cross reactivity and poor protein folding could be the direct consequence of its addition. Putting these finding together makes it tempting to speculate that the inclusion of additional moieties like COMP does not meet the criteria of attaining more robust immune responses. It should be noted that further investigations involving various other antigens should be carried out to be certain about the adverse consequences of COMP inclusion. Our results have revealed that well designed minimal recombinant proteins with desired immunological properties and proper folding could be deemed as adequate immonogenes to produce specific polyclonal antibodies against challenging antigens like hTSH. Moreover, to achieve the highest immunological potency of challenging antigens like hTSH, inclusion of minimal fusion sequences like PII and P30 epitopes of tetanus toxin (with low molecular weight, high potency to help humoral immunity and no humoral immunity against their own sequences) could be of high significance.

ACKNOWLEGEMENTS

The authors wish to thank Tarbiat Modares University and Dezful University of Medical Sciences for supporting the conduct of this research.

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