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Selection of specific inhibitor peptides in enzyme-linked immunosorbent assay (ELISA) of cardiac troponin i using immuno-dominant epitopes as competitor

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Abstract

Human cardiac troponin I (cTni) is the gold marker for early diagnosis of myocardial infarction. In this regard, four immune-dominant epitopes of cTni were predicted and their 3D structures were determined. Thereafter, the competitive performance of the peptides were monitored with the developed polyclonal antibody-based indirect competitive ELISA; a half-maximal

inhibitory concentration (IC50) of 0.49 (µg/ml) and detection limit of 0.037 (µg/ml) were achieved for recombinant cTni. The competitive ELISA determined sensitivity levels of 0.306, 0.141, 0.960, and 0.155 (µg/ml), respectively, for each peptide as competitor. We indicated that two of the selected epitopes have significant sensitivity scales and inhibition ability.

Keywords: cardiac troponin i; enzyme-linked immunosorbent assay; immunodominant epitopes; 3d molecular structure; molecular dynamics, polyclonal antibody

Introduction

The Human Cardiac Troponin I (hcTnI) is a 210 amino acids protein, 23 kDa in molecular weight. As the inhibitory subunit of the troponin complex, hcTnI inhibits actomyosin ATPase activity in the absence of Ca^{2+}.[1] This biomarker is commonly used to diagnose myocardial infarction, micro injury, and acute coronary syndrome (ACS) in patients referring to emergency departments.[2] The American Heart Association (AHA) and European Society of Cardiology (ESC) proposed troponin I as the gold biomarker for early detection of heart attack, especially in myocardial infarction (MI).[3] hcTnI concentration increases drastically about 4-6 h after chest pain occurs and remains high for 14 days.[4] hcTnI is a better biomarker than CKMB or LDH due to its sensitivity and specificity in the early detection of MI and micro injury.[3a, 5] To detect a biomarker in the case of myocardial infarction and micro injury, it is vitally important to have a monoclonal antibody (mAb) with high affinity and specificity against the desired antigen. To this end, it seems more rational to select the elicited antibody employing immuno-dominant peptides rather than whole protein for either of immunization or clone selection. An immuno-dominant peptide encompasses a unique sequence capable of selecting a high affinity mAb. Because hundreds of cell clones are produced in the hybridization and limiting dilution process, selecting

high affinity clones is laborious and time consuming. Using peptides instead of the whole protein may increase the possibility of selecting specific and high affinity clones. Since empirical methods of delving for an immune-dominant epitope could be arduous, costly and time-consuming. In silico prediction of immunodominant peptides would circumvent the drawbacks and would help to have an accurate selection of peptides which are immunologically more potent. Moreover, the study of their structural properties would pave the way to give new insight into hcTnI biology and function. The structural information about the selected peptides would come in handy dealing with the methods of optimizing antibody/peptide interaction affinity during in silico affinity maturation.

The present study used pre-selected epitopes instead of whole hcTnI to evaluate the affinity of each epitope against polyclonal antibodies with indirect competitive ELISA after immunization with whole recombinant hcTnI and identified the best peptide for antibody selection. Initially, the immune-dominant peptides were selected and their structures were studied thoroughly employing an in silico approaches. Thereafter, recombinant human troponin I was used to produce polyclonal antibodies (Ab). Then, each peptide was evaluated to select the best peptide suitable for interaction. These findings may be helpful for the selection of better clones in the process of monoclonal Ab production.

Materials and Methods

In Silico epitope prediction

To select epitopes, the hydrophobicity, antigenicity, flexibility, and structure of the antigens were surveyed with IEDB analysis (http://tools.immuneepitope.org/bcell/) and IMED (http://imed.med.ucm.es/Tools/antigenic.html).

Homology modeling of the epitopes structures

The 3D structures of the predicted epitopes were modeled using the homology modeling approach. To arrive at a proper template, the PDB (http://www.rcsb.org/pdb/home/home.do) was searched to find a 3D structure for hcTnI. Then, Modeller software was employed to model each of the epitopes.

Quality assessment and model refinement

The quality of the modeled structures was assessed using the Prosa server at https://prosa.services.came.sbg.ac.at/prosa.php.

Energy minimization and molecular dynamics (MD)

UCSF Chimera v.1.10.2 was used to perform 1000 steps of the steepest descent followed by 1000 steps of conjugate gradient minimizations on the modeled peptide structures. Thereafter, each peptide was fed to VMD software and prepared for a 1 nanosecond NAMD2 molecular dynamics run solvated in a water cube.

Protein Expression and purification

The full length of the hcTnI DNA sequence was chemically synthesized and cloned into expression vector pET-32a (Biomatik, Canada). The pET-32a-hcTnI vector was transformed to E.Coli Bl21 competent cell and was grown on Luria Bertani (LB) agar containing 100mg ampicillin L^{-1}. A positive colony was propagated at 37^{o}_{C} in LB broth supplemented until the bacterium reached logarithmic growth phase (at OD600 0.6–0.7) and then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). The expression of the his-tagged hcTnI protein was optimized at different IPTG concentrations (0.1, 0.25, 0.5, and 1 mM) and temperatures (18, 25, and 37°C). The his-tagged hcTnI protein was purified in accordance with the manufacturer's

procedures for Ni+–NTA resin-packed columns (Qiagen, Germany). To optimize the purification of hcTnI, different concentrations of imidazole (200, 300, and 500 mM) were used. Protein expression and the purity of the eluted fractions were analyzed by SDS-PAGE (5% stacking gel and 12.5% separating gel). The verification of the recombinant hcTnI was confirmed by Western blot analysis.

Production of polyclonal Ab for hcTnI

The recombinant protein of hcTnI was used to prepare antibodies in New Zealand white rabbits weighing 2–2.5 kg. Multiple-site injection was used to prepare polyclonal antibodies against the hcTnI. Rabbits were immunized with 500µg recombinant protein emulsified in an equal volume of complete Freund's adjuvant (1st day) and 250 µg recombinant hcTnI with incomplete adjuvant (5 injections at a 14-day interval). Serum was harvested from rabbits at 7 days after the final boost, and immune response was monitored by indirect ELISA. The study protocol was approved by the Animal Care and Use Committee (ACUC) of Tarbiat Modares University (TMU).

Indirect ELISA Test

To assess the successful rabbit immunization against recombinant hcTnI, an indirect ELISA test was performed in which 96-well ELISA microplates (Nunc-Maxisorp) were coated with 100 µl of antigen (1µg hcTnI ml-1 diluted in Phosphate Buffered Saline (PBS)) (pH 7.4) and incubated overnight at 4oC. Plates were blocked with 250 µl of blocking solution (0.5% w/v skimmed milk/PBS) and incubated for 2 h at 37oC. After 4 washings with Phosphate Buffered Saline Tween (PBST), 100 µl of rabbit serum (1:500, 1:1000, 1:2000, and 1:4000 v/v) was added to duplicate wells and incubated for 60 min at 37ºC. Non-immunized serum (1:500 v/v) was used for negative control .The plates were washed again with PBST and incubated with 100 µl/well of

a 1:5,000 v/v dilution of HRP conjugated anti-rabbit IgG (Sigma) for 30 min at 37ºC. After 4 washings with PBST, 100 µl of tetramethyl-benzidine (TMB) substrate (Sigma) was added to each well. Plates were then incubated for 15 min at room temperature. The reactions were stopped by adding 50 µl/well of 1M HCl. The absorbance was measured by an ELISA plate reader (Multiskan) at 450/630 nm.

Indirect Competitive ELISA

In order to assess the degree of synthetic epitop reactivity towards the antibody we performed a competitive immunoassay .In the competitive assay, procedures were also basically the same as with the indirect ELISA assay explained in above section, except that after removing the blocking buffer, 50 uL of competitors in diluted antiserum (1:2000 v/v) were added to each well sequentially. The competitors were 4 synthetic peptides in concentrations of 0.01 to 10000 µg/ml. Absorbance was measured at 450 nm using an automatic microplate reader (Multiskan). The inhibition ratio was calculated as %Inhibition = %B/B0, where B is the absorbance for the well containing the competitor and B0 is the absorbance for the well without competitor. The sigmoid curve, regarded as the inhibition curve, was fitted to a logistic equation. Then, half maximal inhibitory concentration (IC50) values, which represent the competitor concentration that leads to a 50% decrease of the maximum signal, were determined.

Results

Selection of epitopes

Using literature available on immunodominant segment of Troponin I molecule and the in silico studies, 4 different peptides, numbered as 1-4, were selected as hcTnI immune-dominant epitopes. Their sequences were as KISASRKLQLKTLLL, RAYATEPHAK, GLGFA, and

TEPHAKKKS, respectively. All of the employed peptides were synthesized and delivered by ChinaPeptides Co. Ltd.

Epitope modeling

The structure of the troponin complex under the PDB ID of 1J1E and 1J1D were used as the template structures of epitope modeling. Modeller software built a model for each epitope and these were employed for subsequent analyses.

Quality analysis

Quality analysis indicates that all of the modeled structures are within the high quality regions of the quality plot. Epitopes 1 to 4 achieved Z scores of -1.46, 1.45, -0.35 and -1.32, respectively.

MD results

Energy minimizations were performed on each model to solve the energy clashes and have a more stable starting conformation for the MD run. The 1 nanosecond MD run let the structure to have its natural behavior in a solvated environment. The resulting structures are depicted in Figure 1.

Optimization of expression

Different concentrations of IPTG were added to the growing culture of containing expression vector. SDS PAGE analysis revealed that the maximum amount of recombinant protein was obtained by adding 1 mM of IPTG at 37^{o}_{C}. In concentrations of 0.1, 0.25, and 0.5, no significant difference in expression was seen. Induced expression increased with 1mM of IPTG which led to maximum expression. The expression of hcTnI was studied at different temperatures in constant IPTG (1 mM optimized); optimum temperature was found to be 18^{oC}. In other temperatures

(25^{o}_{C}, 37^{o}_{C}) no difference in expression was observed. SDS PAGE analysis showed that minimum expression was achieved at 25^{o}_{C} and 37^{o}_{C}, and at 18^{o}_{C} maximum expression was obtained. In other words, maximum expression was obtained at a temperature of 18^{o}_{C} and a 1mM IPTG concentration during 4 h. Figure 2A shows the SDS-PAGE analysis of expression under optimized conditions.

Optimization of purification procedure

Optimization of purification in different concentrations of imidazole resulted in the best elution being with 500 mM of imidazol. Figure 2B shows the SDS-PAGE analysis of purification with 500 mM imidazole. Western blot analysis with anti his-tag antibodies confirmed the purification procedure (Figure 2C).

Antibody production and assessment of antibody titer

The purified human recombinant hcTni was injected into rabbits as described in section 2.6. Blood samples were collected at several points in time from the start of immunization and were tested with ELISA against hcTnI to evaluate the rising of an immune response (Figure 3). An indirect ELISA test of the rabbit serum revealed an increase in reactivity toward hcTnI 5 weeks after the beginning of immunization (Figure 3).

Competitive indirect ELISA

The sensitivity of the immunoassay and the ability of peptide epitops to inhibit antigen antibody interaction was detected under optimal assay conditions and expressed by IC50 value using different peptide sequences (Table 1). The inhibition curve for hcTnI is shown in Figure 3. These results reveal that the IC50 value was 0.490 µg/ml and the limit of detection (LOD) was 0.037 µg/ml, indicating excellent sensitivity for antibody antigen reactivity. The standard curve

exhibited good linearity (R2 = 0.9454) with hcTnI concentrations from 0.01–10 µg/ml (data not shown). Meanwhile, peptides 1, 2, 3, and 4 showed LOD values of 0.05, 0.066, 0.078, and 0.047 µg/ml with IC50 values of 0.306, 0.141, 0.960, and 0.155, respectively. In the cases of peptides 2 and 4, IC50 values were lower than those of other peptides (1, 3) in which the respective LOD values were acceptable and fairly close to the recombinant hcTnI (LOD: 0.037) (Figure 4).

Discussion

Critical steps in polyclonal Ab production are the precise selection of the target antigen and the evaluation of predictions made for its antigenicity. Human cardiac troponin I is a 23 kDa protein that is a monomeric protein composed of five helixes and one turn as its secondary structure. [6] Herein, the authors aimed to evaluate immuno-dominant epitopes of hcTnI for polyclonal antibody evaluation and their use to evaluate peptide immunogenes for clone selection in case of hybridoma production. Immuno-dominant peptides were selected from reports on epitops available in literature and their sequence properties of hydrophobicity, antigenicity, linearity, and the existence of a beta turn were further evaluated. Using BepiPred Linear Epitope Prediction server, maximum antigenicity was observed in a sequence spanning amino acids 1-42 of the hcTnI protein. [7] With the Chou and Fasman Beta-Turn Prediction server, maximum antigenicity was found to be at the N terminal sequence of hcTnI. At the sequence encompassing 1-42, maximum hydrophobicity was found. The structure of the hcTnI is mainly composed of 6 functional segments including: (1) cardiac-specific N-terminal extension (spanning amino acids 1–30), (2) an N-terminal region (spanning amino acids 42–79 that interacts with the C domain of TnC), (3) a TnT-binding region (spanning amino acids 80–136); (4) the inhibitory peptide (spanning amino acids 128–147 capable of interacting with TnC and actin–tropomyosin), (5) the switch or triggering region (amino acids 148–163 capable of binding the N domain of TnC), and

(6) the C-terminal region (amino acids 164–210 that binds actin–tropomyosin and shows the highest sequence conservancy) .[8] The selected epitopes belong to the cardiac-specific N-terminal extension and the region flanking the TnC binding segment. These regions are the sites of various phosphorylation modifications, playing pivotal roles in hcTnI functions. These regions are involved in hcTnI interaction with troponin C leading to their unavailability for the immune system. These results were confirmed by Chou and Ferrieres.[9] Since this region is conceived from the immune system, [10] its exposure to the immune system was expected to elicit high immune responses. Given the aforementioned reasons our epitope selection seems to be rational. However, the immunological studies seem to be halfhearted without accompanying structural data. Thus we performed a through structural analysis to arrive at the structures of the selected epitopes. These structure are vitally important assessing the antigen antibody interactions. Moreover, they could be used to plan logical strategies for monoclonal antibody affinity maturation studies. Knowing the structural intricacies of these epitopes would help to comprehend the network of the amino acids involved in antibody epitope interface.

In this study, the hcTnI gene was cloned into the expression vector pET32a. Different parameters were adjusted to optimize the expression of the recombinant protein. The results showed that the highest yield was obtained in the presence of 1 mM IPTG at 18°C for 4 h and that the expressed protein was localized in the soluble phase.

The sensitivity of the immunoassay was detected under optimal assay conditions and expressed by an IC50 value. IC50 values were lower for peptides 2 and 4 than for other peptides, and the respective LOD values were acceptable and fairly close to the recombinant hcTnI (LOD: 0.037). Increasing sensitivity in the competitive ELISA in peptides 2 and 4 may be caused by the interaction of troponin C with the N-terminal of troponin I. [11]

Conclusions

Results of the current study propose that two sequences (RAYATEPHAK and TEPHAKKKS) have maximum antigenicity because of their involvement in binding with troponin C. This could be rationalized by the fact that this region is not exposed in the immune system. Moreover, these results indicate that in silico predictions could pave the way for better and more accurate research design and results.

Declaration of interest

The authors declare no conflict of interests.

Acknowledgments

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Table 1. Limit of detection (LOD) and IC50 data when partial hcTnI was used as competitive protein and peptides (1, 2, 3 and 4) were used as inhibitors.

Competitor	sequence	LOD(μg/ml)	IC50(μg/ml)	Minimum OD	Maximum OD
hcTnI	1-210	0.037	0.490	0.094	0.964
Peptide 1	40-54	0.050	0.306	0.317	1.007
Peptide 2	31-39	0.066	0.141	0.310	1.087
Peptide 3	87-91	0.078	0.960	0.231	0.988
Peptide 4	27-36	0.047	0.155	0.282	1.014

Figure 1. The schematic diagrams of final structure of the four epitopes are depicted. The structures are numbered according to the epitope numbers.

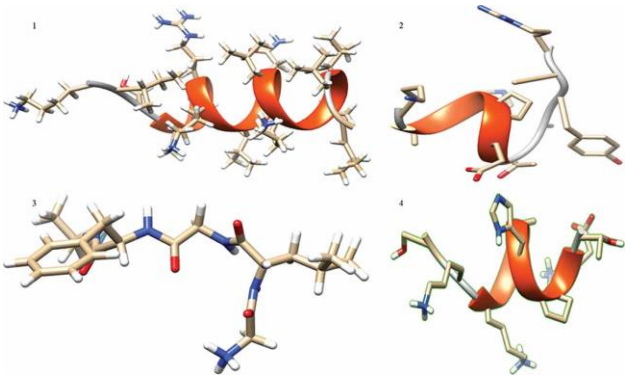

Figure 2. Expression and purification of the recombinant hcTnI. (A) optimization of recombinant protein production at different time with constant IPTG (1 mM) and time (4 h); line 1, protein markers; line 2, 25 $^{o}_{C}$; line 3, 37 $^{o}_{C}$; line 4, 18 $^{o}_{C}$. (B) SDS PAGE of purified protein in optimized conditions (IPTG 1 mM, temperature = 18 $^{o}_{C}$). (C) Western blotting of purified protein hcTnI.

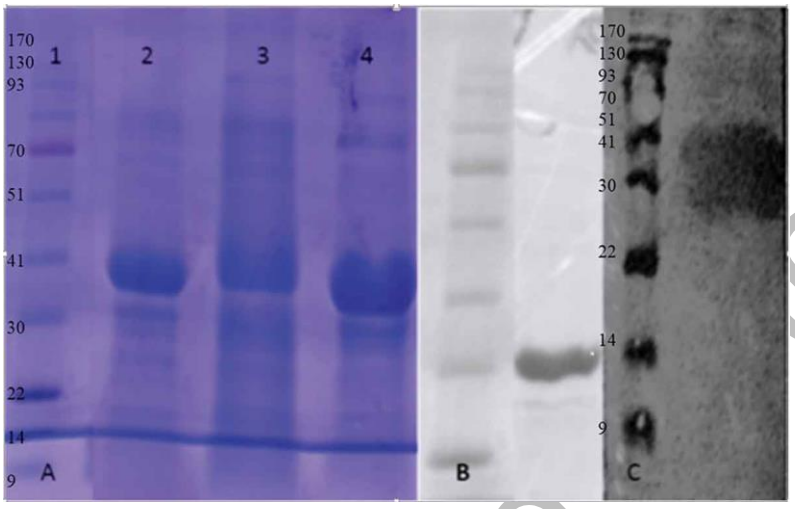

Figure 3. Evaluation of the rabbit immune response raised against recombinant hcTnI. ELISA reactivity test against hcTnI (1 μg/ml), before immunization (Normal Rabbit Serum) and serum samples (diluted to 1:2000 v/v) taken from the immunized rabbit at several points in time. Antibody/antigen interaction was detected by goat anti-rabbit HRP-conjugated antibody.

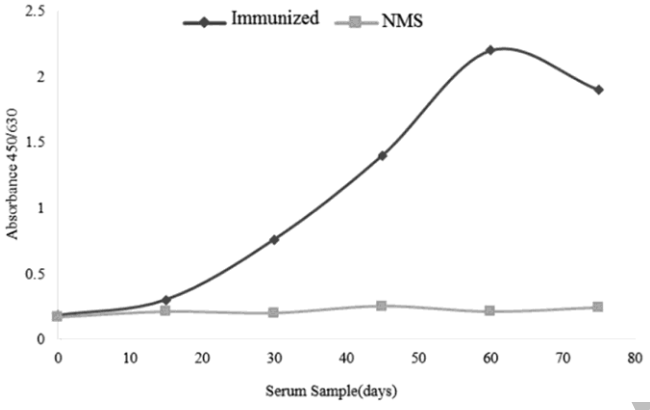

Figure 4. Inhibition curve for indirect competitive ELISA for recombinant hcTnI, peptide 1, peptide 2, peptide 3 and peptide 4.The highest inhibition were found to be exerted by peptide 4 (TEPHAKKKS)