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Development of Innovative Bio-Tools for a cTnI-Detection Assay

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Introduction

Cardiac troponin I (cTnI) constitutes a biomarker for acute myocardial infarction diagnosis [1]. cTnI tests are ELISA-based and require at least two specific antibodies, particularly expensive. Chickens are excellent experimental animals for antibodies' production. Chicken antibodies (IgY) can be isolated from eggs since they pass from serum to egg yolk [2]. Also, chicken produces higher titer antibodies than other animal species with lower cost. Subsequently, the number of experimental animals significantly decreases, while chickens do not forbear painful blood sampling [3]. This study aimed to develop innovative and low-cost specific bio-tools and techniques to detect the cTnI. For this purpose, a series of haptens (consequently possible antigenic epitopes) were selected based on the homology, antigenicity, and hydrophilicity [4-7]. Also, based on our previous studies we exploited two sequential oligopeptide carriers developed in our lab (SOC & CPSOC) [8] to anchor the desired haptens, expecting to enhance in this way the animal immunoresponse but also be usable as adsorbents on ELISA plates during immunoassays. The haptens and peptide carriers were synthesized in SPPS and then linked via thioether bonds to form possible immunogenic conjugates. Experimental animals (laying hens) were immunized with the conjugates. The peptide conjugates were injected into three chickens, following two different approaches that differed in immunogen concentration and the intervals between immunizations. The first approach: 4 injections of immunoconjugate (1 mg/ml)/animal/10 days. The second approach: 3 injections of a mix of immunoconjugates (four immunogenic complexes with a decreasing concentration from 0,3 to 0,1 mg/ml) in one animal in intervals three and five weeks respectively. The antibodies were isolated from their eggs and then purified. The antibodies were tested for their specificity against the peptide conjugates and the cTnI by ELISA assays. Furthermore, an ELISA was utilized to detect cTnI by using commercial anti-cTnI antibodies IgG as capture antibodies and the produced IgY antibodies as detection antibodies.

Results and Discussion

The epitope mapping of cTnI in combination with previous studies, conducted in our lab, resulted in the selection of four peptide sequences which are $cTnI^{19\text{-}31}$, $cTnI^{66\text{-}77}$, $cTnI^{110\text{-}122}$, $cTnI^{118\text{-}131}$. The synthesized conjugates are listed in Table 1. Indicatively the ESI-MS spectra and RP-HPLC chromatograms of the two later conjugates are depicted in Figure 1 and they attest the successful synthesis of the conjugates.

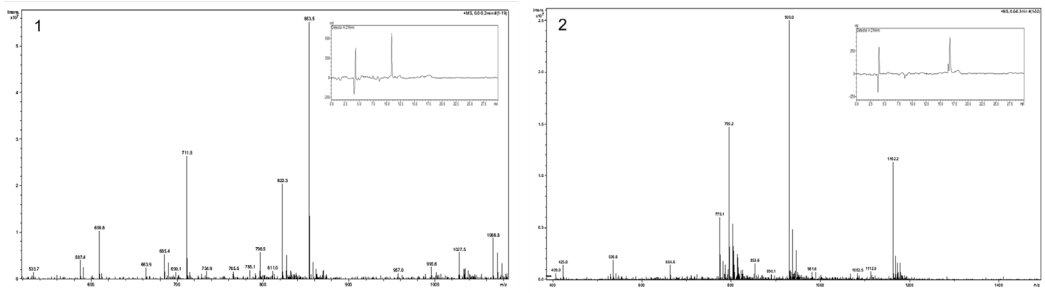

Fig. 1. ESI-MS spectra and RP-HPL chromatograms of peptide conjugates: 1. CPSOC(3,9-Acm;6,12-$cTnI^{66\text{-}77}$) Expected MW: 4263,96 and Found MW: 4262,10 ± 0,61. 2. CPSOC(3,9-Acm;6,12-$cTnI^{110\text{-}122}$) Expected MW: 4646,48 and Found MW: 4644,86 ± 0,25.

Table 1. Peptide conjugates and column that were used for IgY antibodies production & purification, and their abbreviations.

Peptide conjugate / column	*Abbreviation*
CH_3CO-[(K-Aib-C(3,9-CH_2CONH_2; 6,12-CH_2CO-E^{66}RRGEKGRALST^{77}-NH_2)]$_4$-NH_2	CPSOC(3,9-Acm; 6,12-cTnI$^{66-77}$)
CH_3CO-[K-Aib-C(3,9-CH_2CONH_2; 6,12-CH_2CO-R^{110}YDIEAKVTKNIT^{122}-NH_2)]$_4$-NH_2	CPSOC(3,9-Acm; 6,12-cTnI$^{110-122}$)
CH_3CO-[K(R^{19}RRSSNYRAYATE^{31})-Aib-G]$_4$-NH_2	SOC-cTnI$^{19-31}$
CH_3CO-[K(T^{118}KNITEIADLTQKI^{131})-Aib-G]$_4$-NH_2	SOC-cTnI$^{118-131}$
Sepharose-C_3H_6O-S-CH_2CO-Ahx-R^{19}RRSSNYRAYATE^{31}-NH_2	Seph@Ahx-cTnI$^{19-31}$

The IgY antibodies' specificity for the immunoconjugates and the entire protein cTnI was tested by a series of ELISA assays, in which the conjugates or the whole protein (cTnI) were coated to the plates and then the produced antibodies were added. The anti-IgY*HRP was used for signal induction and the samples were measured at 450 nm in an ELISA reader. Antibodies from the same hens before immunizations were used as negative control. Also, ELISA sandwich-type assays were designed and performed to detect the cTnI. For this purpose, anti-cTnI (IgG) commercial antibodies were used as capture antibodies, while the antibodies produced against the mixture of the four immunogenic conjugates were used as detection antibodies.

IgY antibodies' specificity for the conjugates 1. SOC-cTnI$^{19-31}$ and 2. SOC-cTnI$^{118-131}$, which were produced according to the 1st approach are illustrated in Figure 2A. Figure 2 B shows the corresponding results for the antibodies produced against the four conjugates 1. SOC-cTnI$^{19-31}$, 2. CPSOC(3,9-Acm; 6,12-cTnI$^{66-77}$), 3. CPSOC(3,9-Acm; 6,12-cTnI$^{110-122}$), 4. SOC-cTnI$^{118-131}$, namely with the 2nd approach.

The anti-SOC-cTnI$^{19-31}$ IgY antibodies were passed through the synthesized column Seph@Ahx-cTnI$^{19-31}$ and the fractions were used to test the specificity of the purified IgY antibodies for the immunoconjugate SOC-cTnI$^{19-31}$. The results are shown in Figure 2C. The IgY antibodies purified by dialysis were used for comparison.

The results of the ELISA that performed to check the specificity of the isolated IgY antibodies against the whole protein cTnI are depicted in Figure 3A. Also, the purified IgY antibodies with affinity chromatography were tested for their specificity for the cTnI as shown in Figure 3B. The IgY antibodies purified by dialysis and a positive control (anti-cTnI, IgY) were used for comparison.

Figure 3C summarizes the results of the sandwich ELISA that used to detect the cTnI. The specific ELISA was performed by using two different anti-cTnI IgGs as capture antibodies and the produced IgY as detection antibodies. IgY non-specific antibodies were used as negative control. According to the Figure 3C, it is inferred that the antibodies produced against the mixture of the four epitopes function in all cases as cTnI detection antibodies.

In conclusion, the produced anti-cTnI chicken antibodies recognized the immunogenic conjugates and human cTnI, while the produced bio-tools are appropriate for using them in cTnI detection assays. The results of the present work showed that the immunized experimental animals produced high titer and specific antibodies against the immunogenic conjugates, which can detect the cTnI. Also, the produced antibodies purified by dialysis can be utilized without further purification to detect the cTnI. Furthermore, our data indicated that the oligopeptide carriers (SOC & CPSOC) [8] are useful in antibodies' production and ELISA assays by exposing immunogenic peptides, confirming our previous studies. Also, our study suggests that the isolated IgY antibodies from the hens' eggs are suitable and relatively inexpensive tools for developing diagnostic tests. The high efficiency of IgY production significantly reduces the number of experimental animals and the used ones do not get harmed.

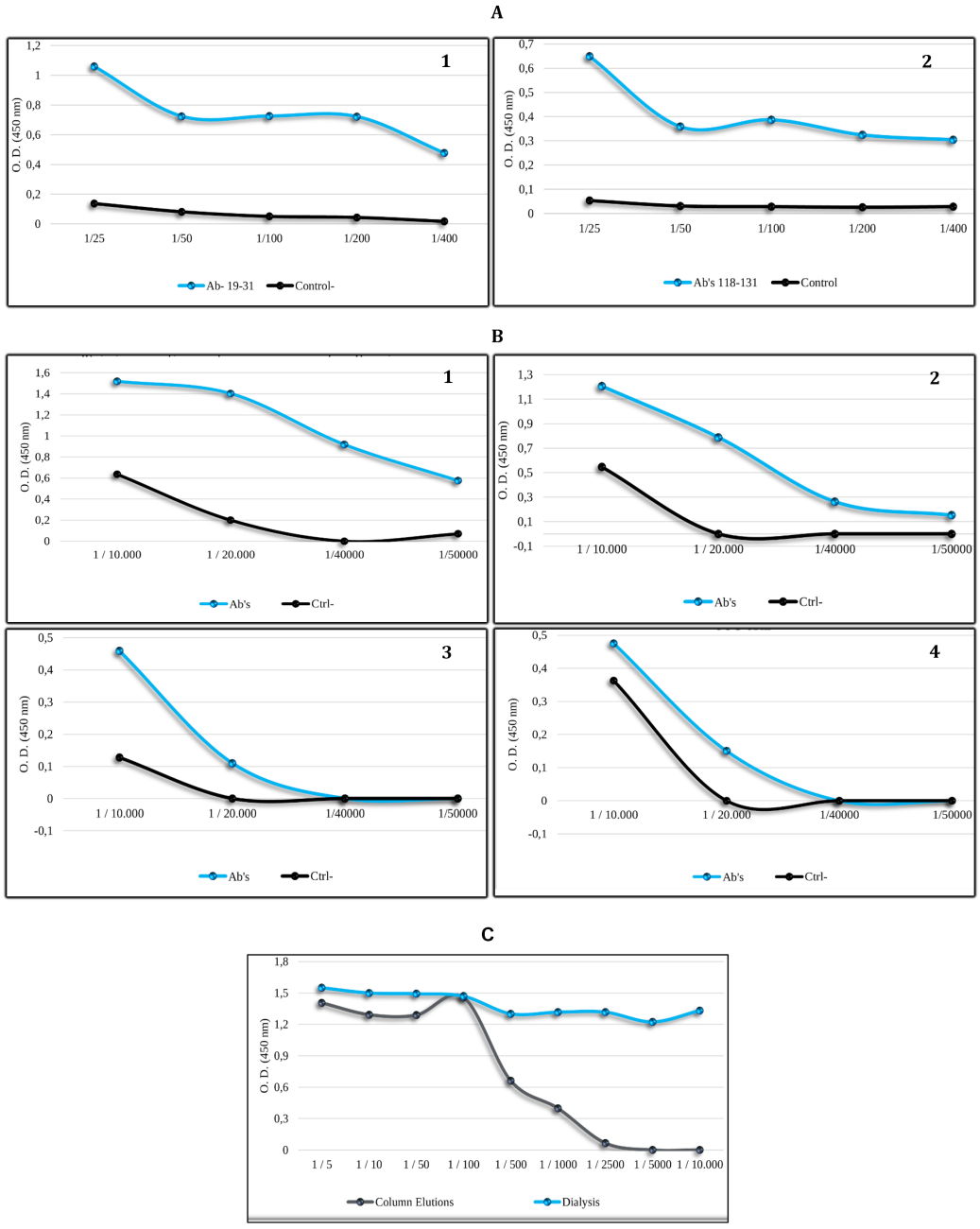

Fig. 2. Specificity of the produced IgY against (A) one epitope/animal (1st approach): 1) SOC-cTnI$^{19\text{-}31}$ & 2) SOC-cTnI$^{118\text{-}131}$ and (B) mixture of four epitopes/animal (2nd approach): 1) SOC-cTnI$^{19\text{-}31}$, 2) CPSOC(3,9-Acm; 6,12-cTnI$^{66\text{-}77}$), 3) CPSOC(3,9-Acm; 6,12-cTnI$^{110\text{-}122}$), 4) SOC-cTnI$^{118\text{-}131}$. (C) Specificity of IgY antibodies, purified with affinity chromatography, for the conjugate SOC-cTnI$^{19\text{-}31}$.

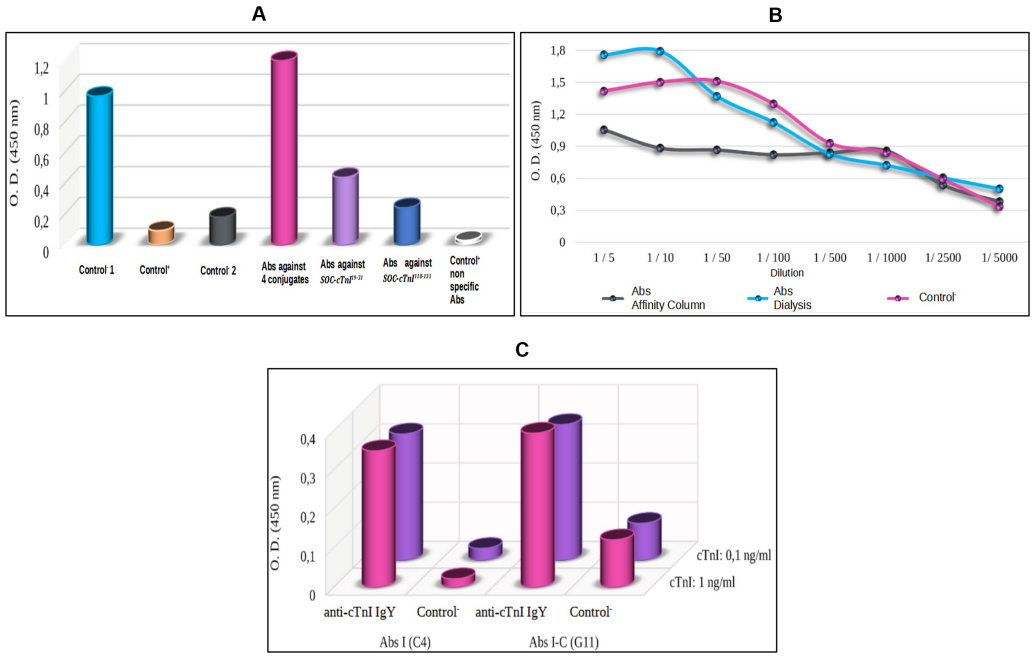

Fig. 3. (A) Results of the ELISA performed for the cTnI detection by the produced IgY. A positive (cyan) & three negative (orange, grey, white) controls were used. (B) Results of the ELISA used to test the specificity of IgY antibodies, purified with affinity chromatography, for the total cTnI. IgY antibodies purified with dialysis were used for comparison and a positive control (pink, anti-cTnI IgY) was used too. (C) Results of the sandwich ELISA used to detect cTnI. Anti-cTnI IgGs were used as capture antibodies and the produced IgY as detection antibodies. IgY non-specific antibodies were used as negative control.

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