Protein-based methods for diagnosing Rheumatoid arthritis

PhD thesis

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1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etology characterized by chronic inflammation of synovial joints. During the formation of the disease, the peptidyl arginine deiminase enzyme is activated and transforms arginyl residues of some proteins into citrullyl residues in a calcium-dependent manner. This modification causes conformational change of the protein, the immune system is activated and attack against its own proteins. The disease is not curable, but using adequate therapy the precession of RA can be slowed down. That is why the early diagnosis of the disease is one of the main research topics. The appearance of autoantibodies to "citrullinated" (deiminated) proteins – anti-citrullinated protein antibodies (ACPAs) – in the serum of patients can be used in the diagnosis of RA [1]. The daily used diagnostic kits – based on these citrullinated proteins (eg. fibrin, vimentin) or citrullinated epitope peptides (eg. filaggrin, collagen) – unfortunately are not enough specific for the disease.

The MTA-ELTE Research Group of Peptide Chemistry, in collaboration with the researchers of the Department of Immunology (ELTE) and the UDEAR Hopital Purpan (Toulouse, France), has been dealing with the epitope mapping of filaggrin and fibrin and with the investigation of new specific diagnostics since 2002 [2-4]. During my thesis work I latch on to this research field.

Beyond the problem of the early diagnosis of RA, we cannot forget about the possibility of curing the disease. The immune response can be decreased by the selective elimination of the autoreactive B-cells. The members of the natural immune system (eg. macrophages) can help to remove the undesirable particles or cells by their effector functions (eg. phagocytosis). On the surface of these cells there are Fcγ receptors, which can indicate the effector functions by immune complex or by Fcγ receptor binding peptides [5-7].

In the second part of my thesis work – in cooperation with the Department of Immunology (ELTE) – I dealt with phagocytosis inducible peptides.

2. Aims

I aimed for the synthesis and immunological studies of various filaggrin és fibrin epitope peptides, which can be useful in the early diagnosis of RA. I have planned the residues as follows:

A) I planned to synthesize biotinylated peptide residues because the base of the developed diagnostic kit was the indirect ELISA method, where the fixation of the peptides to the plate via avidin-biotin interaction.

B) I wanted to know, if the position of the biotin could influence the serum antibody binding, so I planned to synthesize *N*- and *C*-terminal biotinylated epitope peptides.

C) To answer that how can the distance between the epitope and the biotin influence the serum antibody binding, I planned to use two kinds of linkers: the 6-aminohexanoic acid and a water solubility increasing ethylene glycol residue (Ttds = 4,7,10-trioxa-1,13-tridecanediamino succinic acid).

D) To improve the method and increase the affectivity, I planned molecules, which contain four identical epitope peptides fixed on a carrier molecule.

The second aim of my thesis work was to synthesize Fcγ receptor binding peptides, which can be useful to eliminate autoreactive B-cells by inducing phagocytosis. Based on preliminary research I planned to synthesize biotinylated linear and cyclic Fcγ receptor binding peptides. Biotinylation was needed because we wanted to study these peptides by flow cytometry using fluorescein-labeled avidin and avidin-covered beads.

The planned new molecules can be classified in four groups (X = citrulline):

1) Filaggrin epitope peptides and their *N*-, and *C*-terminal biotinylated forms

^{306}SHQESTXGXSXGRSGRSGS^{324} ^{311}TXGRS^{315}

2) Fibrin epitope peptides and their *N*-, and *C*-terminal biotinylated forms

α ^{36}GPXVVEXHQSACKDS^{50} α ^{40}VEXHQSA^{46}, α ^{36}GPXVVEX^{42}

β ^{60}XPAPPPISGGGYXAX^{74} β ^{69}GGYXAXPA^{76}

3) Biotinylated Fcγ receptor binding peptides

FcγRI	Berntzen1	ADGACLRSGRGCGAAK
FcγRIIa	Berntzen2	WAWVWLTETAV
FcγRI	Bonetto	AQVNSCLLLPNLLGCGDDK

4) MAP conjugate containing four identical epitopes

1. generation lysine tree conjugated with four identical fibrin β$^{60-74}$ epitopes

3. Methods

Solid-phase peptide synthesis: The planned new biotinylated filaggrin, fibrin and Fcγ receptor binding peptides were synthesized by solid phase methodology using Fmoc-chemistry. All bioconjugates were characterized by analytical RP-HPLC and ESI mass spectrometry.

Indirect Enzyme-linked Immunosorbent Assay (ELISA): Antibody binding of the synthesized filaggrin and fibrin epitopes was detected by indirect enzyme-linked immunosorbent assay (ELISA) using sera from RA, Systemic lupus erythematosus (SLE) patients as well as healthy individuals.

Electronic cirkular dichroism spectroscopy (ECD): The secondary structure of filaggrin conjugates was investigated by electronic circular dichroism (ECD). We examined the effect on the antibody recognition of the *N*- és *C*-terminal biotinylation, namely the effect of the orientation of the peptides.

Flow cytometry: The phagocytosis inducing affect of the synthesized Fcγ receptor binding peptides and the binding of the MAP conjugates to autoreactive B-cells were studied by flow cytometry.

4. Results and discussion

I synthesized new *N*- and *C*-terminal biotinylated filaggrin and fibrin epitope peptides, the original epitopes as controls and Fcγ receptor binding peptides. During the synthesis of the four identical epitope containing MAP conjugate several problems occurs, the synthesis succeeded partially.

All peptides were synthesized manual or using SYRO automat peptide synthesizer by solid phase methodology using Fmoc-chemistry. All peptides were purified by semipreparative RP-HPLC and the purified compounds were characterized by analytical RP-HPLC and ESI mass spectrometry.

4.1. Antibody recognition of the biotinylated filaggrin epitopes

Antibody binding of the synthesized differently biotinylated filaggrin epitopes was detected by indirect enzyme-linked immunosorbent assay (ELISA) using sera from RA, Systemic lupus erythematosus (SLE) patients as well as healthy individuals.

We wanted to answer the following questions: 1. Is there any difference between the antibody binding of the *N*- and *C*-terminal biotinylated residues? 2. How can the incorporated linkers influence the recognition properties of the epitopes? 3. How specific are these epitopes for RA?

The studies suggest that in case of the filaggrin$^{306\text{-}324}$ epitope region there is no difference between the binding affinity of the *N*- or *C*-terminally biotinylated peptides, while in case of the filaggrin$^{311\text{-}315}$ minimum epitope drastic change occurred: only the *C*-terminally biotinylated epitope peptide could bind serum autoantibody (*Figure1*). The incorporated linkers did not influence the antibody binding and the control sera (healthy subjects and SLE patients) showed no reactivity to any of the peptides. These results indicate that the binding of the *C*-terminally biotinylated epitope peptide (^{311}TXGRS^{315}) and both the *C*- or *N*-terminally biotinylated epitope region peptide (^{306}SHQESTXGXSXGRSGRSGS^{324}) is specific for RA [s1,s2].

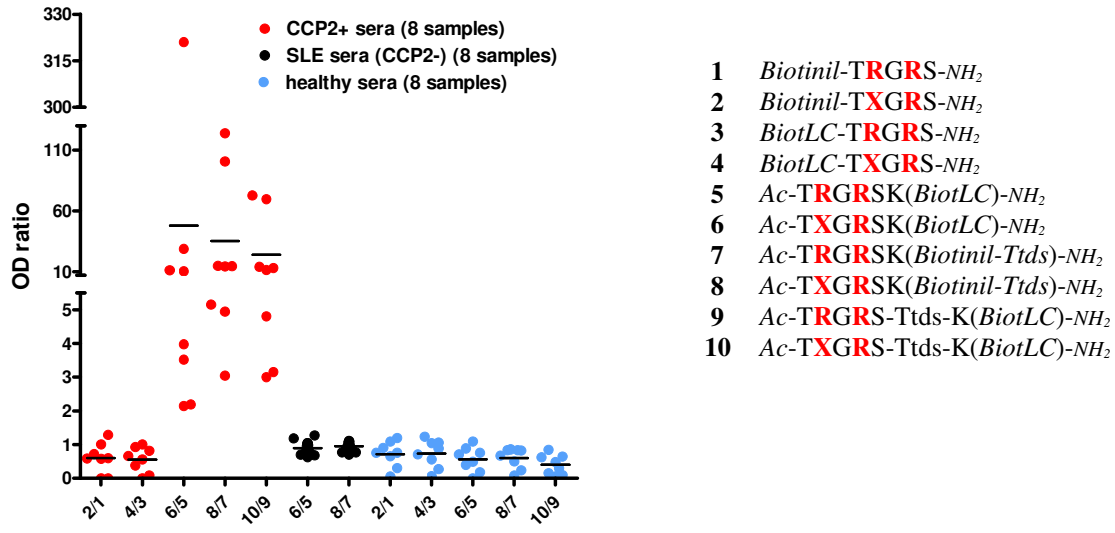

Figure 1. Binding of antibodies from RA sera to peptides containing the minimum epitope (^{311}TXGRS^{315}) of filaggrin as detected by indirect ELISA. OD ratio = OD value of the citrulline containing peptides divided by the OD value of the arginine containing peptides

4.2. Conformation of the filaggrin epitope peptides

Based on the results of the ELISA experiments one question was indicated: what is the reason that in case of the filaggrin$^{311\text{-}315}$ minimum epitope there is a big difference in the serum antibody recognition while in case of the filaggrin$^{306\text{-}324}$ epitope region there is no difference between the binding affinity. Is there a change in the conformation of the peptide or the biotin-avidin complex shades the antibody recognition site? To answer this question I have studied the conformation of the filaggrin epitopes.

The affect of the position of the biotin on the conformation of the filaggrin epitopes was studied by ECD spectroscopy. ECD spectra were recorded in water and in trifluoroethanol (TFE).

I compared the spectra of the arginine and citrulline containing analoges corresponding to the filaggrin$^{306\text{-}324}$ and filaggrin$^{311\text{-}315}$ epitopes. Data suggested the Arg/Cit change does not influence the solution conformation of the peptide conjugates.

I also compared the spectra of the citrulline containing peptides in water and in TFE (*Figure 2 and 3*). Data demonstrated that the presence and position of the biotin moiety has a pronounced effect on the conformation of the filaggrin$^{311\text{-}315}$ epitope core peptides peptide in water and even in ordered structure promoting TFE, while it doesn't alter the secondary structure of the filaggrin$^{306\text{-}324}$ epitope region peptides.

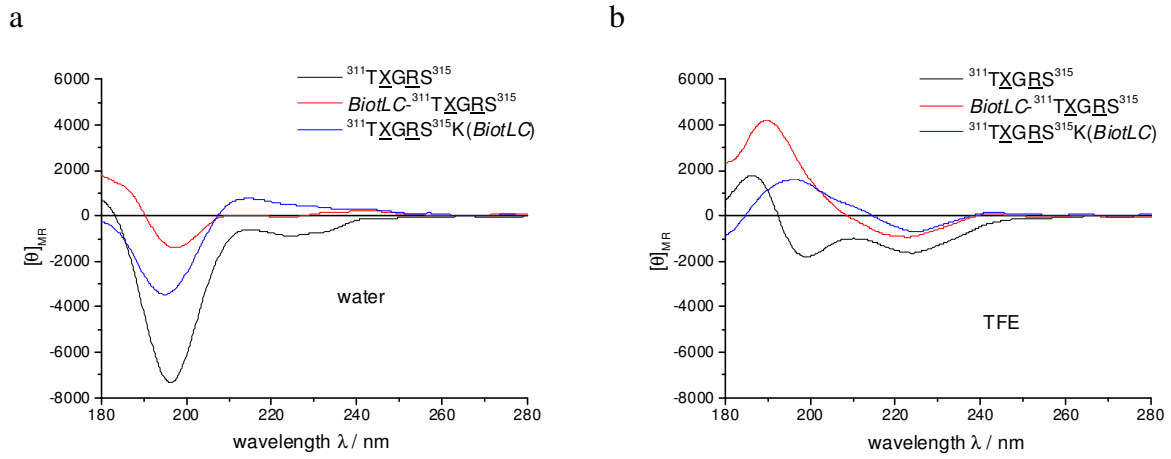

Figure 2. ECD spectra of the ^{311}T**X**G**R**S^{315} filaggrin epitope core and its biotin conjugates (a) in water and (b) in trifluoroethanol. ECD values were given in molar ellipticity, ($[\theta]_{MR}$ / deg cm^2/dmol, ahol MR = mean residue)

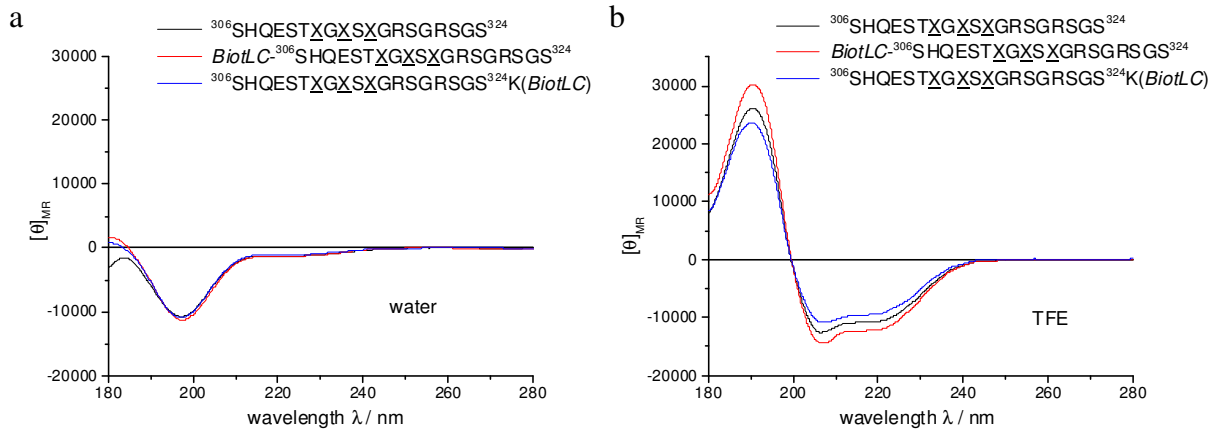

Figure 3. ECD spectra of the ^{306}SHQEST**X**G**X**S**X**GRSGRSGS^{324} filaggrin epitope region and its biotin conjugates (a) in water and (b) in TFE.

Our data indicated that in case of the filaggrin$^{306\text{-}324}$ epitope region only the primer structure of the peptide determines the RA-specific antibody binding. In this case the position of the biotin did not influence the binding, because the epitope core can be found in the middle of the epitope region, in equal distance from both *N*- or *C*-terminal. In contrast with this in case of the filaggrin$^{311\text{-}315}$ minimum epitope peptide both the primer and secondary structure influence the antibody binding. Furthermore, I proved that the positioning of the biotin label on a peptide sequence can profoundly influence its recognition by antibodies thus the sensitivity of an ELISA [s1].

As a final conclusion I demonstrated that the shading effect of the biotin-avidin complex predominates, so the position of the biotin – the orientation of the peptides in the ELISA experiments is determining.

4.3. Antibody recognition of the biotinylated fibrin epitopes

In case of the fibrin epitope peptides the differences in the antibody binding, what was indicated by the position of the biotin, was the same as in case of the filaggrin epitopes. The C-terminal biotinylation is more favorable in case of the fibrin α chain epitopes, while N-terminal biotinylation is more prosperous in case of the fibrin β chain epitopes [s3].

4.4. Reactivity of the identical epitopes containing MAP conjugate with B-cells

The binding of the MAP conjugates to autoreactive B-cells were studied by flow cytometry. Acetylated lysine tree and Streptavidin-FITC were used as controls. Data

demonstrated aspecific binding, because the conjugates and the controls were bound in highest amount than it should have been expected from the B-cell number. These data indicated that these constructions cannot be able to use under these circumstances. In the future it should be worthy to avoid the biotin-avidin system and to use other labeling molecule and other carrier.

4.5. The phagocytosis inducing affect of the Fcγreceptor binding peptides

The phagocytosis inducing affect of the synthesized Fcγ receptor binding peptides were studied by flow cytometry. U937 cells phagocyted similarly the peptide-covered beads, but in a small compass, compared to the controls, so these peptides are not enough affective to reach the aim. In the future study of other Fcγ receptor binding peptides is needed.

5. Summary

During my thesis work I successfully synthesized new biotinylated filaggrin and fibrin epitope peptides. The results of the serum antibody binding assays indicated that the filaggrin$^{311\text{-}315}$, the filaggrin$^{306\text{-}324}$ and the fibrin $\beta^{60\text{-}74}$ epitopes are RA-specific and the C-terminal biotinylated filaggrin epitope peptides and the N-terminal biotinylated fibrin $\beta^{60\text{-}74}$ epitope are useful to investigate a new diagnostic kit. Furthermore I also demonstrated that the orientation of the peptides can profoundly influence its recognition by antibodies thus the sensitivity of an ELISA. Based on the results of this we propose an approach how to identify the best analogue of an epitope peptide of a given sequence for binding studies: biotinylate peptides both *N*-terminal and *C*-terminal, incorporate spacers of different lengths and types, and screen to determine the optimal combination of these elements to develop an efficient and sensitive antibody binding assay.

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s1 **Babos, F.**, Szarka, E., Nagy, Gy., Majer, Zs., Sármay, G., Magyar, A., Hudecz, F. (2013) The role of *N*- or *C*-terminal biotinylation in autoantibody recognition of citrullin containing filaggrin epitope peptides in Rheumatoid arthritis. *Bioconjugate Chem., 24*, 817-827.

s2 Szarka, E., **Babos, F.**, Magyar, A., Huber, K., Szittner, Z., Papp, K., Prechl, J., Pozsgay, J., Nagy, Gy., Rojkovich, B., Kelemen, J., Baka, Zs., Brózik, M., Pazár, B., Poór, Gy., Hudecz, F., Sármay, G. (2013) Recognition of new citrulline-containing peptide epitopes by autoantibodies produced in vivo and in vitro by B cells of rheumatoid arthritis patients. *Immunology,* doi:10.1111/imm.12175.

s3 Cornillet, M., Sebbag, M., Verrouil, E., Magyar, A., **Babos, F.**, Ruyssen-Witrand, A., Hudecz, F., Cantagrel, A., Serre, G., Nogueira, L. (2013) The fibrin-derived citrullinated peptide β60-74$Cit_{60,72,74}$ bears the major ACPA epitope recognised by the Rheumatoid Arthritis-specific anti-citrullinated fibrinogen autoantibodies and anti-CCP2 antibodies. *Ann. Rheum. Dis.,* doi:10.1136/annrheumdis-2012-202868.

Other publications

Baka, Zs., Barta, P., Losonczy, Gy., Krenács, T., Pápay, J., Szarka, E., Sármay, G., **Babos, F.**, Magyar, A., Géher, P., Buzás, E., Nagy, Gy. (2011) Specific expression of PAD4 and citrullinated proteins in lung cancer is not associated with anti-CCP antibody production. *Int. Immunol., 23, 405-414.*

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