Benefits of Immunomagnetic Separation for Epitope Identification in Clinically Important Protein Antigens: A Case Study Using Ovalbumin, Carbonic Anhydrase I and Tau Protein

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Abstract Immunomagnetic separation (IMS) with specific antibody as affinity ligand immobilized on a magnetic carrier has several advantages in comparison with standard column separation procedures. Epitope mapping enabling identification and characterization of protein structures reactive with the antibody represents one possible application of IMS. We used epitope extraction technique based on the proteolytic digestion of the target protein followed by capturing of a specific peptide fragments by the antibody immobilized on the solid phase. Magnetic particles coated with antibody molecules were first incubated with the prepared mixture of peptides. After specific binding of peptide fragments comprising the epitope sequences, the beads were washed to remove non-epitope peptides. Captured epitope-peptides were then eluted in small volume of 0.05% TFA. Elution fractions were finally analyzed without any modification by mass spectrometry. In this work the results and experience gained in epitope mapping of three clinically important proteins (ovalbumin, carbonic anhydrase I and tau protein) are discussed.

Keywords Immunomagnetic Separation, Superparamagnetic Microparticles, Epitope Extraction, Ovalbumin, Carbonic Anhydrase I, Tau Protein

systems usable in a wide range of applications in the biosciences. Moreover, IMS allows the application of crude sample (e. g. cell lysates, blood), it is gentle to target molecules (e. g. protein complexes remain intact), and it doesn't lead to dilution of the sample [1]. Superparamagnetic microparticles (MPs) developing a mean magnetic moment only in the presence of an external magnetic field represent suitable solid phase for IMS.

The magnetizable beads are first coated with the specific monoclonal/polyclonal antibody, allowing search and bind to target molecules/cells, and are then selectively manipulated using magnetic separator. IMS employing superparamagnetic MPs coated with specific antibody has become an essential tool for high-throughput and low-cost enrichment/isolation of various biomolecules and cells from heterogeneous samples. Examples of IMS applications are: (i) extraction of bacterial cells from the food/biological samples followed by detection using real-time PCR [2-7] or mass spectrometry (MS) analysis [8-10], (ii) positive selection/removal of specific cells from blood, bone marrow and other biological samples [11-20], (iii) capturing and detection of clinically important protein biomarkers [21-24], etc. To increase the sensitivity IMS can also be performed in a microfluidic arrangement [25-27] or may be combined with detection using quantum dots (QDs) [28-30].

One possible application of IMS is an epitope mapping. There are several methods available for the identification and characterization of epitopes. Majority of them are based on immunochemical study of the interaction between protein or peptide antigens and antibodies of corresponding specificity. Understanding the antibody/antigen interactions and identification of the epitopes in general are crucial for numerous applications including discovery and development

1. Introduction

The main advantage of immunomagnetic separation (IMS) compared to standard column separation methods, is fast and simple handling of the sample using magnetic forces. It makes this type of separation ideal for automated analysis

of new therapeutics, especially vaccines, and diagnostics. We used epitope extraction technique [31, 32] in combination with MS analysis (Fig. 1). Silica superparamagnetic MPs were used as a solid phase for immobilization of proteolytic enzymes and specific antibodies. It enabled us easy and quick separation of the particles from solution by application of a simple block magnet and easy resuspension by removing of the magnet.

Here we discuss our results obtained using epitope mapping of different clinically important proteins: (i) ovalbumin, the major protein of egg white and a typical representative of food allergens, (ii) carbonic anhydrase I, the zinc metalloenzyme highly abundant in erythrocytes catalysing reversible hydration of carbene dioxide, reliable diagnostic and prognostic indicator in many autoimmune and cancer diseases of humans, and (iii) tau protein, microtubule-associated protein, a major component of paired helical filaments (PHFs) and a biochemical marker for axonal degeneration in Alzheimer's disease (AD).

2. Materials and Methods

2.1. Enzymatic Digestion of Target Protein Antigen

Proteolytic enzymes (3 mg), trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK-trypsin, EC 3.4.21.4, Sigma-Aldrich, St. Louis, MO, USA) or α -chymotrypsin from bovine pancreas, type II (EC 3.4.21.1, Sigma-Aldrich, St. Louis, MO, USA), were immobilized on 1 mg superparamagnetic microparticles

SiMAG-Carboxyl (1 μ m, Chemicell, Berlin, Germany) or carboxylate-modified Sera-Mag® SpeedBeads™ (0.816 μ m, Thermo Fisher Scientific, Waltham, MA, USA) by one-step carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC, 0.12 M) and N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS, 0.02 M) [33, 34], overnight at 4°C in 0.1 M phosphate buffer pH 7.3 under mild stirring (in the case of trypsin in the presence of 2 mM benzamidine as the competitive inhibitor). Resulting proteolytic activity (IU of enzyme/mg of MPs) was estimated using low-molecular weight substrates: N α -benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA, Sigma-Aldrich, St. Louis, MO, USA) for trypsin and N-succinyl-L-phenylalanine-p-nitroanilide (SUPHEPA, Sigma-Aldrich, St. Louis, MO, USA) for α -chymotrypsin.

Albumin from chicken egg white, Grade VII (ovalbumin, OVA, Sigma-Aldrich, St. Louis, MO, USA), carbonic anhydrase I from human erythrocytes (CA I, Sigma-Aldrich, St. Louis, MO, USA) and human, recombinant protein Tau-441 (Enzo Life Sciences, Plymouth Meeting, PA, USA) were first unfolded by 0.1% RapiGest SF (Waters, Milford, MA, USA) combined with reductive alkylation using 50 mM DL-dithiothreitol (DTT) and 100 mM iodoacetamide (IAA) [35-37] in 50 mM ammonium bicarbonate solution. Proteolytic digestion using immobilized enzymes followed in the enzyme : substrate (E : S) molar ratio 1 : 20 for 3 h/overnight at room temperature (RT)/37°C under mild stirring. Protein digests were collected and aliquots were analyzed by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tris–tricine–SDS-PAGE) and/or MS.

Figure 1. Schematic representation of magnetic beads-based epitope extraction

2.2. Immunocapturing of Specific Epitope-peptide Fragments

Specific antibodies: (i) anti-OVA isolated from chicken ovalbumin antiserum produced in rabbit (Lot No. A30-111-3, Bethyl Labs., Montgomery, TX, USA) [36], (ii) anti-CA I isolated from serum of patient with multiple myeloma after high-dose therapy (HDT) with autologous stem cell transplantation (ASCT) treatment (National Cancer Institute, Bratislava, Slovakia) [37], and (iii) anti-Tau protein isolated from serum of rabbit immunized with Tau-441 (Prague Psychiatric Center, Prague, Czech Republic) [38] were covalently bound through carbohydrate moieties to 1 - 5 mg superparamagnetic microparticles SiMAG-Hydrazide (1 μ m, Chemicell, Berlin, Germany). Antibodies were firstly oxidized with 20 mM sodium meta-periodate (NaIO_4), oxidation was stopped by the addition of ethylenglycol and unreacted oxidation reagent was removed by desalting and buffer exchange through a MicroSpin G-25 Column (GE Healthcare, Buckinghamshire, UK). Then immobilization was carried out overnight in 0.1 M acetate buffer pH 4.6 with 0.2 M NaCl or 0.1 M phosphate buffer pH 7.0 at RT with continuous stirring. The remaining active groups of the carrier were blocked with 0.1 M D-glyceraldehyde (Sigma-Aldrich, St. Louis, MO, USA) [36-38].

Peptide fragments of OVA, CA I or Tau-protein (5 - 375 μ g) were applied to the equilibrated magnetic MPs (0.5 - 1 mg) with immobilized specific antibody. After 1.5 - 4 h incubation at RT under mild stirring, the non-bound peptides were removed by intensive washing with 0.1 M phosphate buffer pH 7.0 with 0.2 M/1 M NaCl, 0.01 M phosphate buffer pH 7.0 and distilled water. Immunospecifically captured peptides were subsequently eluted with 0.05% trifluoroacetic acid (TFA, Fluka, Buchs, Switzerland) - 3 $\times$ 200 μ L, 15 minutes, RT, mild stirring. Obtained elution fractions were concentrated before MS analysis using speed-vac [36-38].

2.3. Mass Spectrometric Analysis

Peptide fractions were analyzed using MALDI-MS (MALDI-TOF system Voyager-DE STR and MALDI-TOF/TOF system 4700 from Applied Biosystems, Foster City, CA, USA; LTQ Orbitrap XL-MALDI MS system from Thermo Scientific, Waltham, MA, USA) and/or LC-MS/MS (nanoAcquity UPLC and Q-ToF Premier/Q-TOF Ultima API, Waters, Milford, MA, USA) measurement [36-38].

The obtained MS/MS data were processed using the PepSeq script in MassLynx 4.0 (Waters, Milford, MA, USA) or the ProteinLynx Global Server v. 2.4 (Waters, Milford, MA, USA). Analysis of position and accessibility of experimentally detected peptides in the original protein structures was performed using different bioinformatic tools (e. g. Clustal 2.0.1, PISA server, CCP4) [37].

The results of magnetic beads-based epitope extraction experiment can be influenced by many factors. There are several parameters that should be optimized to minimize non-specific interactions and maximize sensitivity and accuracy of the analysis. We should take into account: (i) type of magnetic MPs (size, core/shell material, surface active groups, etc.), (ii) proteolytic digestion parameters (type of proteolytic enzyme, protein modification before digestion, enzyme : substrate ratio, conditions of digestion – time, temperature, buffer composition, etc.), (iii) immunocapturing parameters (type of antibody – monoclonal/polyclonal, antibody : peptides ratio, conditions of immunocapturing – time, temperature, buffer composition, etc.), (iv) final washing and elution (composition of individual solutions – addition of salt, acid/alkaline elution, number of individual steps, etc.). In this paper optimized protocols for three different protein antigens are mentioned (OVA, CA I, Tau protein). They are applicable in general also for other proteins, but it is necessary to keep in mind that every biological system is individual and further optimizations may be needed, in order to improve the results, according to the properties of the specific components. Examples of the effect of various parameters on the results of selected analyzes are shown in the following text.

Arrangement of magnetic beads-based epitope extraction experiment is one of the parameters that can significantly affect the final results. In the case of ovalbumin we compared results obtained using batch-wise arrangement in 2 mL microtube and microfluidic arrangement using special polydimethylsiloxane (PDMS) device enabling fixation and self-organization of magnetic MPs in chain-like columns [34, 39]. Similarly to chromatographic separations, analogous interactions occur on the surface of magnetic particles. A self-organized plug of biofunctionalized magnetic beads in microchannel of microfluidic device profits mainly from an even larger surface-to-volume ratio, an enhanced interaction of the specific surface with passing analytes, and an improved recuperation of reaction products [40].

As can be seen in Fig. 2A microfluidic arrangement provided really better results than batch-wise arrangement. In both cases MS spectra of the elution fractions repeatedly demonstrated the presence of one abundant peptide (m/z : 1345.8, missed cleavages: 0) but the spectra obtained using the microfluidic device were more conclusive and clear without presence of other contaminant peptides [36]. It may be due to the continuous flow during the microfluidic experiment that suppressed non-specific sorption and weak interactions. The amino acid sequence of this detected tryptic peptide was confirmed by tandem MS sequencing with Q-TOF (HIATNAVLFFFGR corresponding to the 371-382 segment of ovalbumin) (Fig. 2B). Identification of epitopes within the allergen molecules represented by ovalbumin can be useful mainly for the development of specific immunotherapy tools such as safe epitope-based peptide vaccines [41].

3. Results and Discussion

Figure 2. Epitope mapping analysis of OVA: (A) MALDI-TOF mass spectra (MALDI-TOF/TOF system 4700) of third elution fractions (0.05% TFA) obtained using SiMAG-Hydrazide microparticles with anti-ovalbumin polyclonal antibodies - microfluidic vs. batch-wise arrangement; (B) MS/MS spectrum (Q-TOF Ultima API) of isolated epitope-peptide OVA fragment (m/z 1345.75), fragment ions (y-series) are labeled (determined sequence: HIATNAVLFFGR, score: 78.49) [36]

For proteolytic cleavage we prefer enzymes immobilized to magnetic particles, which can be in contrast with soluble form of the enzyme removed from the mixture by a magnetic field after finishing the reaction and can be used repeatedly. Each protease has its own specific recognition sites where the peptide bond is cleaved (e. g. trypsin only cleaves at lysine or arginine residues). The problem is when cleavage sites are involved in epitope sequence. Then specific peptide after the cleavage can not be recognized by the antibody. For this reason it is advisable to try more enzymes differing in specificity (e. g. trypsin, α -chymotrypsin, proteinase K, pepsin [36], Arg-C, Lys-C endoproteases) and obtained results compared to each other. In experiment focused on identification of CA I immunodominant epitopes recognized by specific autoantibodies isolated from serum of patient with multiple myeloma after HDT/ASCT therapy we applied immobilized trypsin and α -chymotrypsin. LC-MS/MS analysis of elution fractions repeatedly revealed presence of four tryptic CA I specific fragments. Three of them were confirmed also by chymotrypsin digestion (Tab. 1) [37].

Table 1. Tryptic fragments of CA I repeatedly identified in elution fractions by LC-MS/MS (nanoAcquity UPLC with Q-ToF Premier), MC = missed cleavages, *confirmed by chymotrypsin digestion [37]

Mass	Position	MC	Peptide sequence
2256.0428	58-76	0	EIINVGHFSFVNFDNDNR*
1929.0076	40-57	0	HDTSLKPISVSYNPATAK
1580.7914	214-227	0	ESISVSSEQLAQFR*
1186.6864	138-149	0	ADGLAVIGVLMK*

It is also advantageous to combine the results of MS analysis with some bioinformatic tool enabling an accurate determination of the epitope sequence within the peptide fragment. For example bioinformatic analysis of solvent accessible surface area (ASA) of individual residues of experimentally detected tryptic fragments using PISA server allowed us to identify amino acids within the CA I structure (SLKPI, NVGHS, DGLAV and SSEQL) which are accessible for possible antibody binding and may form the epitope (Fig. 3) [37]. Knowledge of CA I immunodominant

epitopes could be useful in theoretical and applied biomedical research of various autoimmune and cancerous diseases, mainly for different diagnostic (e. g. immunoassay reagent development) or therapeutic purposes (e. g. active/passive immunization).

Figure 3. Epitope mapping analysis of CA I: analysis of solvent accessible surface area of individual residues of detected peptide fragments within the CA I structure (PDB code 1AZM) using PISA server, the location of each peptide in the CA I structure is represented in a corresponding color [37]

The results of epitope extraction experiment may also be influenced by physico-chemical properties of studied molecule. For example proteins susceptible to form aggregates represented by Tau protein may exhibit unexpected behavior and complicate the analysis. During epitope mapping of Tau protein using specific antibodies isolated from hyperimmune rabbit serum we observed one intense peak in the MS spectrum (m/z : 1980.1, position: 299-317, missed cleavages: 0, sequence: HVPGGGSVQIVYKPVDSLK) which we regarded as

specifically captured fragment comprising an epitope [38]. But when we repeated the experiment with monoclonal anti-Tau antibody (clone 7E5) of known epitope (RGAAPPGQKGQA included in tryptic fragment with m/z 1423.7), non-specific fragment with m/z : 1980.1 also occurred with relatively high intensity in the MS spectrum (Fig. 4) [42]. This fragment is located at the microtubule assembly domain and sequence VQIVYK (position: 306-311) between repeat R2 and R3 is considered to be aggregation motif with tendency to form β -structures [43, 44] which could be related to higher non-specific sorption [45].

To avoid such inaccurate interpretation of the results it is necessary to include in each experiment appropriate negative control and minimize the possibility of non-specific interactions. The most common ways to suppress non-specific sorption include: (i) low-molecular reagents blocking the remaining reactive groups on the MPs surface (e. g. D-glyceraldehyde for hydrazide functional groups [36-38], ethanolamine and Tris for carboxylate functional groups [45]), (ii) MPs surface modification by protein/polymer layer (e. g. BSA [46], PEG [47, 48], hyaluronic acid [49]), (iii) adding low levels of salt, chaotropic reagent or detergent in the binding and/or washing buffer (e. g. NaCl, sodium thiocyanate, SDS, urea [45]). The specificity of binding can be confirmed also by capturing in the presence of a large excess of other proteins or peptides (e. g. tryptic fragments of 1% BSA [37]) or using another antibody (differing in specificity or non-specific [45]). Knowledge of major epitopes of Tau protein could help to reveal the mechanism and significance of autoimmune reaction ongoing within the pathogenesis of AD.

Figure 4. Epitope mapping analysis of Tau protein: mass spectra (MALDI LTQ Orbitrap XL) of elution fractions (0.05% TFA) obtained using SiMAG-Hydrazide microparticles with polyclonal anti-Tau protein antibodies isolated from hyperimmune rabbit serum and monoclonal anti-Tau protein antibodies 7E5 [38, 42]

4. Conclusions

Magnetic-beads based epitope extraction technique can be advantageously applied for wide range of proteins of various origin (e. g. animal, plant, bacterial, recombinant). Which was demonstrated in this work by successful analysis of three various clinically important protein molecules - ovalbumin, carbonic anhydrase I and Tau protein. Moreover, it is a procedure that is robust, with the exception of the final MS detection instrumentally undemanding, and can be performed in any bioanalytical laboratory.

Although the procedure is quite simple and generally applicable, we may encounter various pitfalls in specific cases, which are necessary to solve and optimize. To achieve the most sensitive and accurate results using this method under optimal conditions we can try different experimental arrangement (e. g. batch-wise, microfluidic), combine MS results with some bioinformatic tool (e. g. analysis of solvent accessible surface area), minimize non-specific sorption (e. g. modification of MPs surface or buffer composition) and/or include some negative control (e. g. MPs with non-specific ligand).

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