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Epitope mapping of allergen ovalbumin using biofunctionalized magnetic beads packed in microfluidic channels
The first step towards epitope-based vaccines

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ABSTRACT

Specific allergen immunotherapy is frequently associated with adverse reactions. Several strategies are being developed to reduce the allergenicity while maintaining the therapeutic benefits. Peptide immunotherapy is one such approach. Methods for the simple and rapid identification of immunogenic epitopes of allergens (i.e. allergenic epitopes) are ongoing and could potentially lead to peptide-based vaccines. An epitope extraction technique, based on biofunctionalized magnetic microspheres self-organized under a magnetic field in a channel of a simple microfluidic device fabricated from polydimethylsiloxane, was applied in the isolation and identification of prospective allergenic epitopes. Similarly to chromatographic column separations, the easily replaceable plug of self-organized beads in the channel benefits especially from an even larger surface-to-volume ratio and an enhanced interaction of the surfaces with passing samples. Ovalbumin, the major protein of egg white and a typical representative of food allergens, was selected as the model molecule. Highly resistant ovalbumin was at first efficiently digested by a magnetic proteolytic reactor with trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone and the second step, i.e. capture of allergenic epitopes from the mixture of peptides, was performed by a magnetic immunoaffinity carrier with orientedly immobilized rabbit anti-ovalbumin IgG molecules. Captured peptides were released with 0.05% trifluoroacetic acid. The elution fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The peptide fragment of ovalbumin HIATNAVLFFGR (*m/z*: 1345.75, position: 371–382) was identified as a relevant allergenic epitope in this way. Such a microfluidic magnetic force-based epitope extraction technique applied in the epitope mapping of ovalbumin has the potential to be a significant step towards developing safe and cost-effective epitope-based vaccines.

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1. Introduction

Adverse reactions to food are relatively common and represent a particular problem for infants and children (reported incidences of 2–8% compared with 1–2% in adults) [1]. An adverse food reaction is a clinically abnormal response to an ingested food or food additives. IgE-mediated food hypersensitivity (type I) accounts for the majority of well-characterized food allergic reactions, although non-IgE mediated immune mechanisms (type III, type IV hypersensitivity—IgG_4) are also responsible for a variety of disorders associated with adverse food reactions [2,3].

Progress in the identification and structural characterization of food allergens will improve the diagnostics and therapy of food allergies. Until now, avoidance is the only rational therapy for food allergy. However, certain factors (e.g. allergy to peanuts, nuts, fish, milk, egg, wheat and soybeans) place some individuals at increased risk of more severe anaphylactic reactions, therefore new types of immunotherapy vaccines specifically for food-induced anaphylaxis are coming into focus as new therapeutical concepts [2,4].

Specific allergen immunotherapy (SIT, specific immunotherapy, hyposensitization) induces a shift in the Th2/Thl balance [5]. The efficacy of specific whole allergen immunotherapy for the treatment of allergic diseases has been demonstrated in numerous

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clinical studies [6,7]. However, due to the use of extracts containing native allergen molecules capable of cross-linking allergen-specific IgE, SIT is associated with a relatively high incidence of adverse events [8]. Several strategies are being developed to reduce the allergenicity of therapeutic substances, while maintaining their benefits. Peptide immunotherapy is one such approach. Short peptide sequences, comprising the immunogenic epitopes of major allergens, are unable to crosslink allergen-specific IgE molecules on basophils and mast cells [9]. It is assumed that immunotherapy with the precise epitope would suppress the generation of adverse reactions. Real-time and cost-effective methods for the simple identification of the epitopes in a number of major food allergens are under development and provide the prospect of safe peptide-based vaccines [8].

A chip-based epitope extraction technique, already validated in our laboratory for low-molecular weight antigens [10], has been fully tested for the rapid and low-volume structural characterization of high-molecular antigens, represented by the food allergen ovalbumin. The epitope mapping algorithm consists of the following steps: (1) digestion of the target antigen by a soluble proteolytic enzyme or enzyme covalently bounded to the solid phase, (2) capture of immunogenic/allergenic epitopes by immunoaffinity chromatography, (3) mass spectrometric analysis of the captured peptides and their identification using an appropriate database [11].

Our strategy was based on a combination of a simple microfluidic device fabricated from polydimethylsiloxane (PDMS) [12,13] with biofunctionalized magnetic microspheres integrated into the microfluidic channel. The most efficient and simple orientation was to arrange the magnets in a repulsive conformation, with the polarisation at a slight angle to a straight channel. The particles self-organize in chain-like columns along the direction of the channel. This simple device contains one inlet connected by tubing to a 500 μL microsyringe and the flow rate of the mobile phase is controlled by a peripheral on-line syringe pump. The most important advantages of using microfluidic systems are known to be in particular the possibility of using minute quantities of sample and reagents (down to picoliters), relatively fast reaction time when molecular diffusion lengths are of the order of the microchannel dimension and a large surface-to-volume ratio, offering an intrinsic compatibility between the use of a microfluidic system and surface-based assays [14]. Magnetic microparticles ("beads") offer a large specific surface for chemical binding, fast and simple handling and in particular can be magnetically manipulated using permanent magnets or electromagnets, independently of normal microfluidic or biological processes [15]. Similar to chromatographic separations, analogous interactions occur on the surface of magnetic particles. A self-organized plug of biofunctionalized beads in an open microchannel profits 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 [14].

2. Experimental

2.1. Chemicals and reagents

Albumin from chicken egg white, Grade VII (ovalbumin, OVA), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.22.2, 12,700 IU mg^{-1} solid), DL-dithiothreitol (DTT), iodoacetic acid (IAA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), benzamidine, *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), D-glyceraldehyde, Tween 20 and *o*-phenylenediamine (OPD) were produced by Sigma–Aldrich (St. Louis, MO, USA). *N*-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and trifluoroacetic acid (TFA) were purchased from Fluka (Buchs, Switzerland). RapiGestSF was supplied by Waters (Milford, MA, USA). Chicken ovalbumin antiserum (produced in rabbit, Lot No. A30-111-3) was obtained from Bethyl Labs. (Montgomery, TX, USA). Perloza MT 500 (80–100 μm) was produced by Severočeské Chemické Závody (Lovosice, Czech Republic). Silica superparamagnetic non-porous microparticles (1 μm) with carboxyl and hydrazide moieties (SiMAG-Carboxyl and SiMAG-Hydrazide) were supplied by Chemicell (Berlin, Germany). The specific secondary antibody SwAHu/IgG-Px was obtained from Sevapharma (Prague, Czech Republic). Albumin from bovine serum, fraction V, pH 7.0 (BSA) was obtained from AppliChem (Darmstadt, Germany). The Micro BCA (BCA = bicinchoninic acid) protein assay reagent kit was produced by Pierce (Rockford, IL, USA). Proteomix calibration mixture PepMix 4 (Proteomix) and 2,5-dihydroxybenzoic acid (DHB) were purchased from LaserBio Labs. (Sophia Antipolis, France). All other chemicals were of reagent grade.

2.2. Equipment

Magnetic separator (Dynal, Carlsbad, CA, USA), UV/Vis spectrophotometer Libra S22 (Biochrom, Cambridge, UK), microfluidic device fabricated in PDMS kindly provided by Institute Curie (Paris, France), syringe pump (kdScientific, Holliston, MA, USA), Mini-PROTEAN 3 electrophoresis system, BioLogic LP system low-pressure chromatograph and 10 × 1 cm column (Bio-Rad, Hercules, CA, USA), matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) system Voyager-DE STR and MALDI-TOF/TOF-MS system 4700 (Applied Biosystems, Foster City, CA, USA), Q-TOF Ultima API (Waters, Milford, MA, USA).

2.3. Methods

2.3.1. Preparation of proteolytic reactor for digestion of ovalbumin

The coupling of trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK-trypsin, 12 mg) to magnetic SiMAG-Carboxyl microparticles (3 mg) using EDAC (0.12 M) and sulpho-NHS (0.02 M) in one step was adapted from Staros [16] and Bilkova et al. [12]. Immobilization was carried out overnight at 4 °C in 0.1 M phosphate buffer pH 7.3 with benzamidine (2 mM) as the competitive inhibitor. The proteolytic activity (IU of enzyme/mg of carrier) was estimated with the standard low-molecular substrate BAPNA [17]. The prepared reactor was stored in aqueous solution (0.1 M phosphate buffer pH 7.3) containing sodium azide and benzamidine (2 mM) at 4 °C.

2.3.2. Tryptic digestion of ovalbumin

Ovalbumin (1.5 mg, 1 mg mL^{-1}) was either dissolved in ammonium bicarbonate with 8 M urea and unfolded by reductive alkylation using DTT and IAA [17] or dissolved in 0.2% RapiGest buffer and unfolded according to the manufacturer's recommended procedure. The solution of unfolded protein (1.5 mg, 1 mg mL^{-1}) digested batch-wise was mixed with the proteolytic reactor (3 mg, molar ratio E:S of 1:10) and incubated for 3 h at room temperature. The solution of unfolded protein (0.5 mg, 1 mg mL^{-1}) for on-chip digestion was injected by the syringe pump at various flow rates (15–120 μL h^{-1}) into the channel of the device (Fig. 1) filled with the proteolytic reactor (1 mg, molar ratio E:S of 1:10) [12]. Protein digests were collected and aliquots were analyzed by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tris–tricine–SDS-PAGE) and/or MALDI-TOF-MS.

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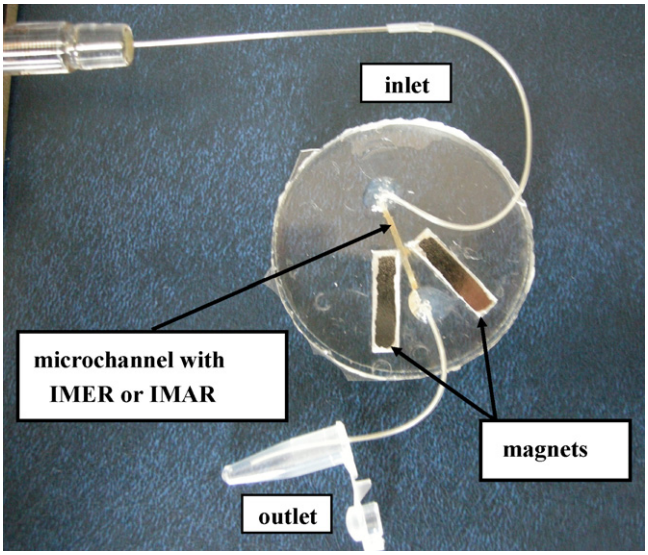

Fig. 1. μ-Chip utilized for proteolytic digestion and subsequently specific immunocapturing: provided by Institute Curie (France), fabricated by rapid prototyping and PDMS technology [18], the channel is 1 mm wide by 250 μm high and 2 cm long, there is silicone tubing at the inlet and the outlet of the channel to inject and collect the solutions, permanent magnets inserted in the cut-outs of the PDMS-block make an angle of 20° with the channel, both magnets have dimensions of 4 mm × 4 mm × 16 mm with a magnetic remanence of 1.3 T at the pole and a polarisation in the longest dimension (IMER, immobilized enzyme reactor; IMAR, immobilized affinity reactor).

2.3.3. Isolation of anti-ovalbumin antibodies by affinity chromatography

Ovalbumin (10 mg) was immobilized to a Perloza MT 500 carrier (5 mL) according to Turkova et al. [19]. The prepared carrier (0.5 mg of ligand/1 mL of carrier) was packed in a column and equilibrated with 0.1 M phosphate buffer pH 7.5 with 0.15 M NaCl (1.5 mL min^{-1}). After the sample was applied (1.3 mL of chicken ovalbumin antiserum, 0.75 mL min^{-1}), the carrier was extensively washed with 0.1 M phosphate buffer pH 7.5 with 0.15 M NaCl (1.5 mL min^{-1}). Captured anti-ovalbumin IgG antibodies were eluted by low pH (0.1 M glycine buffer pH 2.8 with 0.15 M NaCl, flow rate 1.0 mL min^{-1}). The eluted fractions were immediately neutralized with 1 M Tris.

2.3.4. Estimation of relative IgG binding efficiency by BCA protein assay

Magnetic microparticles (100 μg) with immobilized IgG molecules was mixed with the working reagent according to the manufacturer's instructions. The mixture was gently stirred for 2 h at 37 °C. The absorbance of the supernatant at 570 nm was measured after cooling to room temperature.

2.3.5. Estimation of relative IgG binding efficiency by secondary anti-IgG antibody with enzymatic detection

Magnetic microparticles (100 μg) with immobilized IgG molecules was mixed with 1.5 mL of blocking buffer containing 1% BSA in phosphate-buffered saline (PBS) pH 7.4 and stirred for 1 h at room temperature. The beads were then incubated for 1 h at room temperature while stirred with 1 mL of specific secondary antibody (porcine anti-IgG/Px) diluted 1:30,000 with PBS pH 7.4. The microparticles were subsequently washed with 0.1 M acetate buffer pH 4.0 with 0.5 M NaCl (3 mL) and 0.1 M acetate buffer pH 5.5 with 0.15 M NaCl (6 mL) and then incubated for 10 min at room temperature while stirred in the dark with 1 mL of substrate solution containing 0.1% BSA, 0.05% Tween 20, 0.003% H_2O_2 and 0.01% OPD. The absorbance of the supernatant at 405 nm was measured.

2.3.6. Preparation of immunoaffinity carrier for capturing the allergenic epitope

Isolated anti-ovalbumin IgGs were used for oriented immobilization through carbohydrate moieties to magnetic SiMAG-Hydrazide microparticles (5 mg). The coupling method was adapted from the manufacturer's procedure. Antibodies were oxidized with sodium *meta*-periodate. Oxidation was stopped by the addition of ethylenglycol and unreacted oxidation reagent was removed by desalting and buffer exchange through a Sephadex G-25 column. The washing and coupling buffer was modified (0.1 M acetate buffer pH 4.6 with 0.2 M NaCl) and immobilization was carried out overnight at room temperature with continuous stirring. The remaining active groups of the carrier were blocked by incubation with 0.1 M D-glyceraldehyde in coupling buffer at room temperature for 1 h while stirred. The prepared carrier was stored in aqueous solution (PBS pH 7.4) containing sodium azide at 4 °C.

2.3.7. Epitope extraction method in batch-wise arrangement

Tryptic peptides of ovalbumin (1.5 mg, 1 mg mL^{-1}) were applied to the immunoaffinity carrier (4 mg), equilibrated with 10 mL of PBS pH 7.4. After 1.5 h incubation at room temperature, the non-bound peptides were removed by washing the carrier with 10 mL of 0.1 M phosphate buffer pH 7.0 with 0.2 M NaCl, 5 mL of 0.1 M phosphate buffer pH 7.0 with 1 M NaCl and 5 mL of 0.01 M phosphate buffer pH 7.0. Immunospecifically captured peptides were subsequently eluted with 0.05% TFA (3 × 200 μL). The carrier placed in the microreaction cup was gently mixed for 15 min at room temperature. The three elution fractions obtained were concentrated to 40 μL, collected and stored for mass spectrometric analysis. The carrier was equilibrated with 10 mL of PBS pH 7.4.

2.3.8. Chip-based epitope extraction method

Tryptic peptides of ovalbumin (200 μg, 1 mg mL^{-1}) were applied to the immunoaffinity carrier (1 mg) integrated into the channel of the PDMS chip (Fig. 1) and equilibrated with PBS pH 7.4 (100 μL, 500 μL h^{-1}). The capturing proceeded at flow rate of 50 μL h^{-1} and room temperature. The non-bound peptides were removed by washing the carrier with 0.1 M phosphate buffer pH 7.0 with 0.2 M NaCl (50 μL, 500 μL h^{-1}), 0.1 M phosphate buffer pH 7.0 with 1 M NaCl (50 μL, 500 μL h^{-1}) and 0.01 M phosphate buffer pH 7.0 (50 μL, 500 μL h^{-1}). Immunospecifically captured peptides were subsequently eluted with 0.05% TFA (4 × 50 μL, 25 μL h^{-1}). The four elution fractions obtained were concentrated to 10 μL, collected and stored for mass spectrometric analysis. The carrier was equilibrated with PBS pH 7.4 (200 μL, 500 μL h^{-1}).

2.3.9. Mass spectrometric analysis

Fractions were analyzed using MALDI-TOF-MS and/or MALDI-TOF/TOF-MS techniques. A solution of 2,5-dihydroxybenzoic acid (DHB) (10 mg mL^{-1} of 20% acetonitrile in water/0.1% TFA) was utilized as the matrix for sample preparation. Aliquots (1 μL) of sample and matrix solution were mixed on the hydrophobic MALDI plate and allowed to dry. Calibration was performed with the standard proteomix calibration mixture PepMix 4 (Proteomix), which includes five individual peptides covering the mass range 500–3500 Da. Detected ions (m/z) were compared with the theoretical values (m/z) of specific ovalbumin tryptic fragments using the PeptideMass tool of the ExPASy proteomics server [20]. The peptide sequence was confirmed by tandem MS sequencing on a quadrupole time-of-flight (Q-TOF) mass spectrometer. The fragmentation pattern of the peptide was compared to the theoretical pattern of the studied sequence using the PepSeq script in MassLynx 4.0 (Waters, Milford, MA, USA).

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3. Results and discussion

The aim of this study was to develop a simple microanalytical system for the structural characterization of allergen molecules. Almost all allergens identified so far are proteins or glycoproteins in nature. Moreover, the surface structure (topology) is the most relevant for antibody binding. Therefore, a lot of research has been focused on determining allergenic structures and epitope mapping. Molecular-level information on the structure of allergens indicates that allergens are significantly heterogeneous protein structures and that there is no particular amino acid sequence which is responsible for allergenicity [21]. Therefore, the information gained from detailed structural, functional and immunochemical studies of allergens would greatly improve our understanding of the underlying allergic disease mechanisms, and our ability to handle these diseases, both in a diagnostic and a therapeutical sense [22].

To obtain information on linear, conformational and/or discontinuous epitopes, various approaches have been described. These methods include X-ray analysis, limited proteolysis and epitope footprinting, epitope excision and epitope extraction for linear epitopes, and probing the surface accessibility of residues by differential chemical modifications of specific amino acid side chains or by differential hydrogen/deuterium exchange of the protein backbone amides for conformational and discontinuous epitopes. We decided to apply the unique benefits of magnetic microspheres combined with a microfluidic device and the methodological approach was based on the principle of the epitope extraction technique [11] in combination with MALDI-TOF mass spectrometry [23].

First of all, magnetic particles with immobilized TPCK-trypsin were utilized for the digestion of ovalbumin, as a model allergen, and subsequently magnetic particles with immobilized polyclonal anti-ovalbumin antibodies were utilized to isolate specific peptides (i.e. allergenic epitopes), in either a batch-wise arrangement or as the integral parts of a microfluidic device (Fig. 1).

Ovalbumin, as a typical representative of food allergens, is highly resistant to enzymatic digestion [24]. Such properties as its intramolecular disulfide bond, glycosylation and the compact structure of the globular protein (Table 1) associated with digestion resistance could be related to its allergenicity [25]. Therefore, ovalbumin was unfolded by reductive alkylation using DTT and IAA and/or RapiGest reagent to make it more susceptible to enzymatic proteolysis (see Section 2.3.2, Fig. 2). To digest this allergen efficiently and obtain a peptide mixture consisting of the desired fragments, proteolytic enzymes immobilized on magnetic microparticles were applied. In comparison to the standard procedure using a soluble form of the enzyme with the usual molar E:S ratio of 1:50, proteolytic reactors with a molar E:S ratio of 1:10 offer the benefits of a reduction in digestion time, absence of autolytic fragments in the peptide mixture and low frequency of missed cleavages. Additionally, the proteolytic reactors provide suitable properties such as high proteolytic activity, low non-specific adsorption, high operational and storage stability and a low tendency of the microspheres to aggregate [12].

Table 1
Model allergen ovalbumin

Allergen nomenclature	Gal d 2	[41]
Molecular mass	44.0–45.0 kDa	[42]
Isoelectric point	4.6	[43]
Disulfide bridges	1 disulfide bond (73–120)	[44]
Glycosylation	Carbohydrate content 3.2%	[45]
	1 *N*-glycosylation site: Asn-292	[44]
Phosphorylation	Phosphate content 1.73 mol/mol ovalbumin	[46]
	2 phosphorylation sites: Ser-68, Ser-344	[47]
Biological function	Storage protein of egg white (60–65%)	[48]
	Belongs to serpin family: active site 352–353	[49]

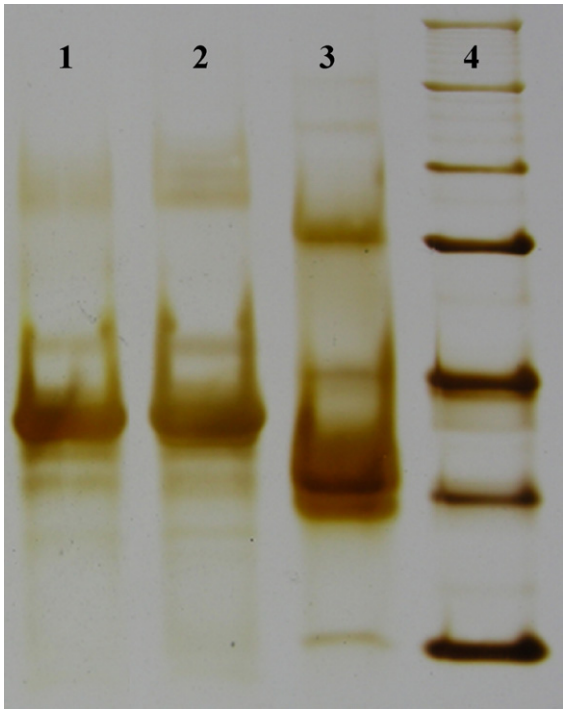

Fig. 2. Tris–tricine–SDS-PAGE: gel—10% T, 3% C (MW 5–100 kDa), detection with silver staining, positions: (1) ovalbumin unfolded by urea, DTT and IAA, (2) ovalbumin unfolded by RapiGest, (3) native ovalbumin, and (4) molecular marker (10–250 kDa).

Table 2
Tested magnetic proteolytic reactors for digestion of ovalbumin

Immobilized proteolytic enzyme	Magnetic microparticles	Determinated specific activity	Specificity/optimal pH	Theoretical number of fragments (missed cleavages: 0)[a]
TPCK-trypsin, 12,700 IU mg^{-1} (Sigma–Aldrich)	SiMAG-Carboxyl 1 μm (Chemicell)	350 IU mg^{-1} of carrier	Arg, Lys/8.0–8.5	24
Proteinase K, 52 IU mg^{-1} (Sigma–Aldrich)		10.4 IU mg^{-1} of carrier	Aliphatic and aromatic residues/7.5–9.0	18 (most below 1 kDa)
α-Chymotrypsin, 50 IU mg^{-1} (Sigma–Aldrich)		16.8 IU mg^{-1} of carrier	Trp, Tyr, Phe, Leu, Met/7.5–8.5	34
Pepsin, 4,550 IU mg^{-1} (Sigma–Aldrich)		nd	Hydrophobic, aromatic residues/2.0–4.0	23 (most below 1 kDa)

nd = not detected.
[a] Using the PeptideMass tool of the ExPASy proteomic server [20].

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Table 3
Magnetic microparticles applied for preparation of bioactive reactors

Microparticles	Composition	Coupling procedure	Ligand
SiMAG-Carboxyl, 1 μm (Chemicell, Germany)	Core: maghemite, matrix: silica, non-porous	Carbodiimide method [12,16]	TPCK-treated trypsin (350 IU mg^{-1} of carrier)
SiMAG-Hydrazide, 1 μm (Chemicell, Germany)		Site-directed method through carbohydrate moieties [26]	Anti-OVA antibodies (10 μg mg^{-1} of carrier)

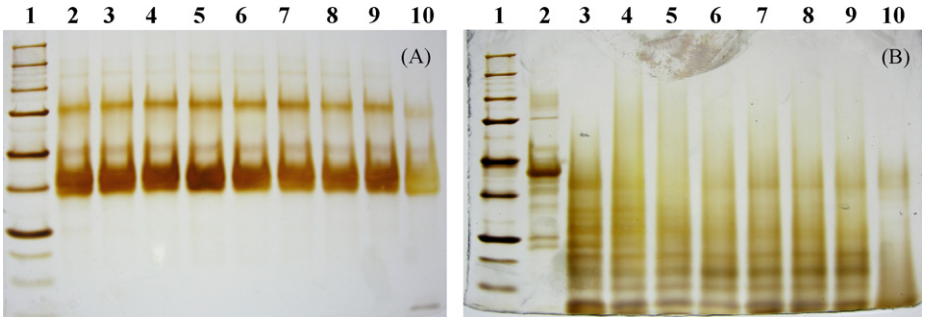

Fig. 3. Tris–tricine–SDS-PAGE: gel—10% T, 3% C (MW 5–100 kDa), detection with silver staining, positions: (1A, 1B) molecular marker (10–250 kDa), (2A) non-digested native ovalbumin, (3A) native ovalbumin digested by TPCK-trypsin proteolytic reactor—15 min, (4A) 30 min, (5A) 60 min, (6A) 90 min, (7A) 120 min, (8A) 150 min, (9A) 180 min, (10A) 24 h; (2B) non-digested ovalbumin unfolded by RapiGest, (3B) ovalbumin unfolded by RapiGest digested by TPCK-trypsin proteolytic reactor—15 min, (4B) 30 min, (5B) 60 min, (6B) 90 min, (7B) 120 min, (8B) 150 min, (9B) 180 min, and (10B) 24 h.

Proteolytic enzymes such as TPCK-trypsin, proteinase K, α-chymotrypsin and pepsin were tested and their efficiency of digestion was monitored. The reactor with immobilized TPCK-trypsin, which exhibits a high specific activity and has irreversibly inhibited chymotrypsin activity, was applied in the routine digestion of ovalbumin (see Section 2.3.2) due to the following observed results: (1) low optimal pH 2–4 and no detected specific activity after the immobilization of pepsin to SiMAG-Carboxyl magnetic microparticles, (2) lower specific activity of immobilized α-chymotrypsin and (3) high rate of extremely short peptide fragments provided by proteinase K (Table 2). TPCK-trypsin was immobilized to SiMAG-Carboxyl magnetic microparticles (Table 3) via a one-step carbodiimide method using EDAC and sulpho-NHS (see Section 2.3.1). The carbodiimide method is a binary covalent binding system and therefore guarantees a good reproducibility of the immobilization. Carbodiimides react with the terminal carboxylate-groups on the magnetic beads to form highly reactive *O*-acylisourea derivatives, which readily react with the amino-groups of the ligands. The final activity of the trypsin reactor (350 IU/mg of carrier) was determined with the low-molecular substrate BAPNA. This reactor can be utilized repeatedly, the enzyme activity after ten utilizations decreased only to 60% of the initial activity. Proteolytic digestion of the model substrate, the food allergen ovalbumin, was carried out at room temperature and with a molar enzyme-to-substrate ratio of 1:10 either in a batch-wise arrangement or with the trypsin reactor as an integral part of a microfluidic device (Fig. 1). The efficiency of tryptic cleavage was confirmed by Tris–tricine–SDS-PAGE (Fig. 3) and MALDI-TOF-MS analysis (Fig. 4). The results of Tris–tricine–SDS-PAGE (Fig. 3A) demonstrate the high resistance of the native protein to digestion, even after 24 h only partial digestion of ovalbumin was observed (position A-10). With unfolded molecules (Fig. 3B), intermediate peptides were found as early as after 15 min of digestion (position B-3).

The specific anti-ovalbumin IgG molecules used in the preparation of the magnetic immunosorbent were isolated from rabbit serum by affinity chromatography with ovalbumin as the ligand (see Section 2.3.3). The carrier with ovalbumin was prepared by

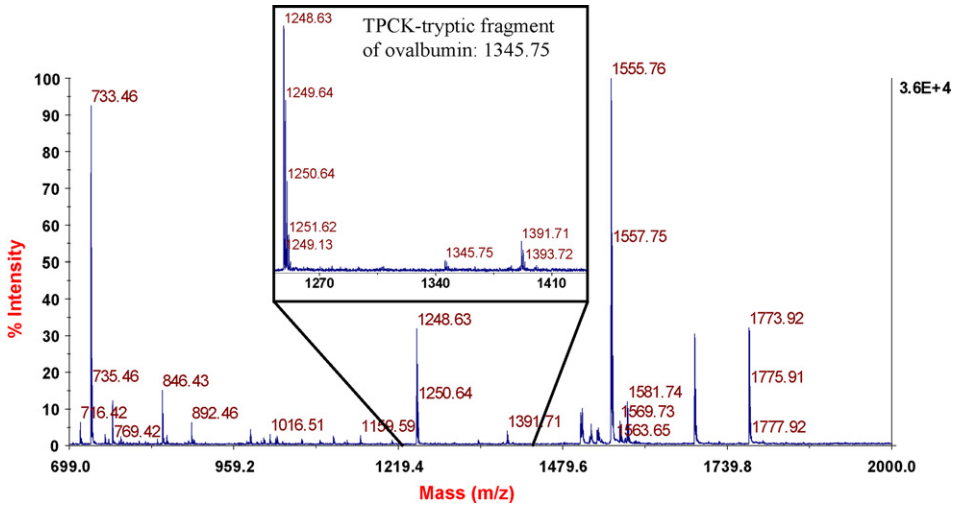

Fig. 4. MALDI-TOF mass spectrum of ovalbumin unfolded by RapiGest digested by TPCK-trypsin proteolytic reactor.

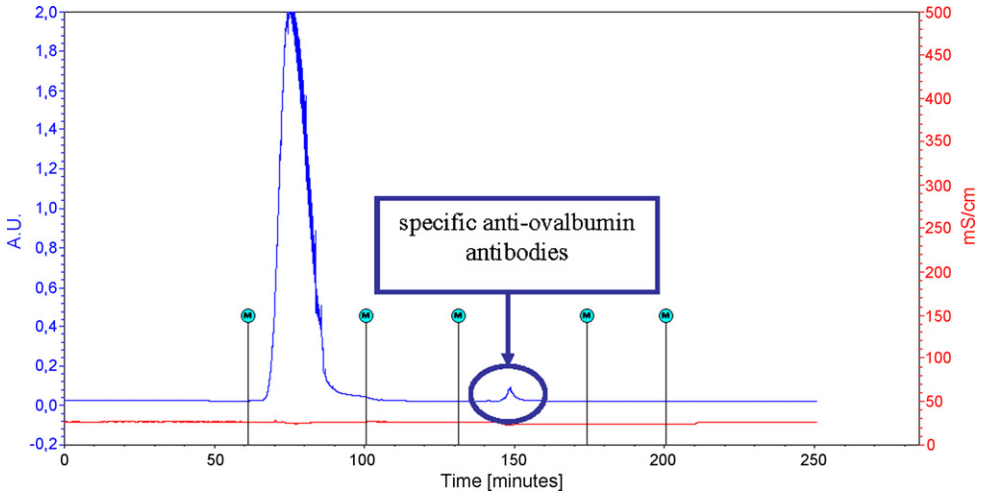

Fig. 5. LC chromatogram of specific anti-ovalbumin antibodies isolation from rabbit serum using an ovalbumin affinity column (see Section 2.3.3): absorbance profile at 280 nm (upper line), conductivity profile (lower line), Perloza MT 500 (5 mL) with ovalbumin (2.5 mg) as ligand in 10 × 1 cm column (Bio-Rad, USA), M, mark of buffer and/or flow rate changing.

a standard methodology according to Turkova et al. [19] based on periodate oxidation of the cellulose carrier and reduction of the Schiff base to a secondary amine by sodium cyanoborohydride. Macroporous bead cellulose (Perloza MT 500, 80–100 μm) with extremely low non-specific sorption was used as the solid phase for the covalent coupling of ovalbumin. The binding capacity of this bioaffinity carrier (10 nmol/mL of carrier) was estimated by the change in absorbance of the ligand solution before and after coupling. The chromatogram (Fig. 5) shows the elution of specific anti-OVA antibodies with the glycine buffer pH 2.8 providing sufficient elution power to release the IgG molecules without any adverse effects on their binding activity. The extremely low pH of the elution fractions was immediately adjusted to a neutral level with 1 M Tris. The purity of the eluted fractions was verified by SDS-PAGE electrophoresis.

In order to prepare a magnetic immunoaffinity carrier for capturing specific ovalbumin peptides, various magnetic microparticles differing in their functional moieties (carboxyl, amino, hydrazide, PGL = polyglutaraldehyde, cyanuric) were tested. Their relative IgG binding efficiency was estimated either by BCA protein assay or with a secondary anti-IgG antibody with enzymatic detection (see Sections 2.3.4 and 2.3.5). BCA protein assay provides a quantitation of the total protein by using bicinchoninic acid (BCA) as the detection reagent for Cu^{+}, which is formed when Cu^{2+} is reduced by a protein in an alkaline environment. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+}). The method, based on the utilization of a secondary antibody to detect an antibody immobilized on microparticles as the solid phase, is a modification of the enzyme-linked immunosorbent assay (ELISA) technique. Microparticles without coupled antibodies were used as negative controls in these methods. The absorbance values for each type of microparticle were compared.

The best results were obtained with hydrazide-derivatized beads (Table 4), therefore SiMAG-Hydrazide magnetic microparticles (Table 3) were utilized in the preparation of the immunoaffinity carrier (see Section 2.3.6). The IgG molecules are in this case immobilized in a specific orientation via their carbohydrates. Formyl groups on the carbohydrate chains generated by oxidation with sodium periodate form stable hydrazone linkages with the hydrazide groups of the carrier. This immobilization approach, with the covalent binding of IgG molecules via a structure far from their binding sites offers the perfect conditions for interaction with antigen or antigenic peptides [26]. The binding specificity of the newly prepared immunosorbent was verified with both native and unfolded forms of ovalbumin. Subsequently, the magnetic immunoaffinity carrier with a binding capacity of 0.2 nmol/mL of carrier was utilized for capturing specific ovalbumin peptides.

This second step of the epitope extraction technique, the isolation of allergenic epitopes, was carried out in either a batch-wise arrangement (see Section 2.3.7) or as an integral part of a microfluidic device (see Section 2.3.8, Fig. 1). The reagents for adsorption, washing and elution were identical for both techniques. The unbound peptides are washed out by the equilibration buffer. This buffer enriched with NaCl was applied to remove non-specifically adsorbed peptides. The composition of the mobile phase during the elution step was optimized with respect to the subsequent MS analysis (see Section 2.3.9). 0.05% TFA, which is compatible with MALDI-TOF-MS, has been shown to be suitable for the elution of this antigen from the immunosorbent. When the isolation was performed in the microchip, the reaction conditions were optimized, especially flow rates of the equilibration buffer (500 $\mu L\,h^{-1}$), sample solution (50 $\mu L\,h^{-1}$) and elution buffer (25 $\mu L\,h^{-1}$). The chip was maintained at a constant temperature between 22 and 25 °C for the assay. After the elution of immunospecifically captured peptides by the low pH of the mobile phase, the immunosorbent was either

Table 4
Relative estimation of IgG binding efficiency on magnetic microspheres differing in functional moieties by specific secondary antibody and BCA protein assay

Functional moiety	Secondary antibody absorbance (405 nm)	BCA protein assay absorbance (570 nm)
Carboxyl	0.685	0.509
Amino	0.305	0.479
Hydrazide	1.166	0.533
PGL	0.824	nd
Cyanuric	0.969	nd

nd = not detected.

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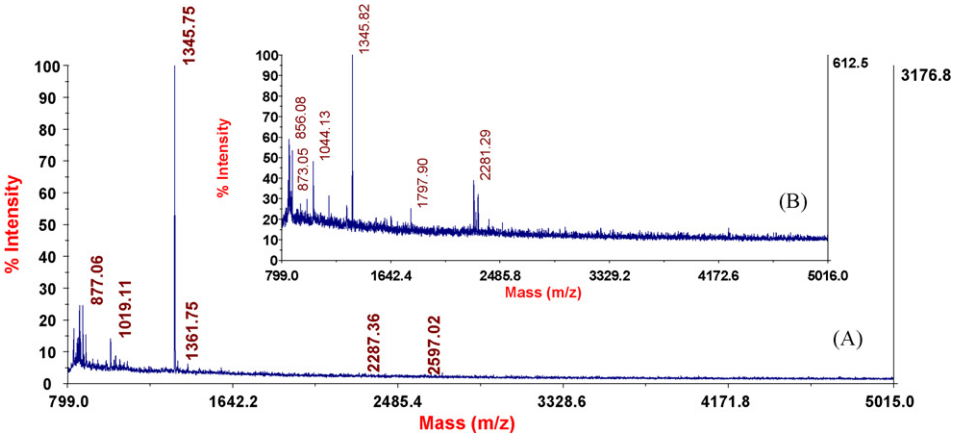

Fig. 6. MALDI-TOF mass spectra of 3rd elution fractions (0.05% TFA) from SiMAG-Hydrazide microparticles with anti-ovalbumin polyclonal antobodies: (A) chip-based immunocapturing (see Section 2.3.8); (B) batch-wise arrangement (see Section 2.3.7).

equilibrated with phosphate buffer for a subsequent immunoassay or disposed of and the device prepared for the next application.

We have identified the peptide, among the trypsin-derived ovalbumin fragments, which displays preferential binding affinity to the utilized anti-OVA polyclonal antibodies. The ions in the MALDI-TOF mass spectra of the elution fractions (Fig. 6) repeatedly demonstrated the presence of one peptide (*m*/*z*: 1345.75, missed cleavages: 0). The detected ions (*m*/*z*) were compared with the theoretical values (*m*/*z*) of specific ovalbumin tryptic fragments using the PeptideMass tool of the ExPASy proteomics server [20]. It was confirmed that the isolated peptide is a specific fragment of ovalbumin. More obvious spectra were obtained with the ovalbumin unfolded by RapiGest buffer and the results provided with the microfluidic device were more conclusive than those obtained with the batch-wise arrangement (Fig. 6B). As can be seen in Fig. 6A, only one high intensity peak corresponding to a specific ovalbumin peptide was detected. The amino acid sequence of the isolated peptide was confirmed by tandem MS sequencing with Q-TOF (Fig. 7). The fragmentation pattern of the peptide was compared to the theoretical pattern of the studied sequence using the PepSeq script in MassLynx 4.0 (Waters, Milford, MA, USA). This sequence (HIATNAVLFFGR) corresponding to the 371-382 segment of ovalbumin includes eight hydrophobic, two polar and two basic amino acid residues and its theoretical isoelectric point (p*I*)/molecular weight (MW) was estimated to be 9.76/1345.57.

A number of studies have reported the partial T and B cell and/or IgE epitope mapping of ovalbumin using various methodological approaches based on the utilization of chemical [27,28] and enzyme-digested [28,29,30] fragments or synthetic peptides [29,31-38]. Our findings are in agreement with results obtained by Honma et al. [29], using immunoblot and histamine release assays, demonstrating the region comprising residues 347–385 of ovalbumin molecule as allergenic epitope. Based on the crystal structure of ovalbumin [39], this fragment (347–385) is located on the surface of the molecule and thus it might be reactive with IgE antibodies in patients with egg allergy [29]. It is known, that the most accessible surfaces of any globular protein appear to be potentially immunogenic. In addition, IgE antibodies recognize determinants associated not only with the conformational structure but also with the primary structure. It has been reported, that treatment of ovalbumin with carboxymethylation, 6 M urea or heating at 95 °C had no significant effect on its binding capacity to human IgE derived from egg-allergic patients' sera [40], which indicates that anti-ovalbumin antibodies recognize mainly the sequential epitopes. This fact supports our analytical strategy to digest ovalbumin at first and then, by means of specific anti-ovalbumin antibodies, to identify the immunogenic sequential epitopes. Kahlert et al. [38] used panel of monoclonal antibodies against native ovalbumin to analyze allergenic epitopes and the results demonstrate that most of the monoclonal antibodies and patients' IgE bind to the frag-

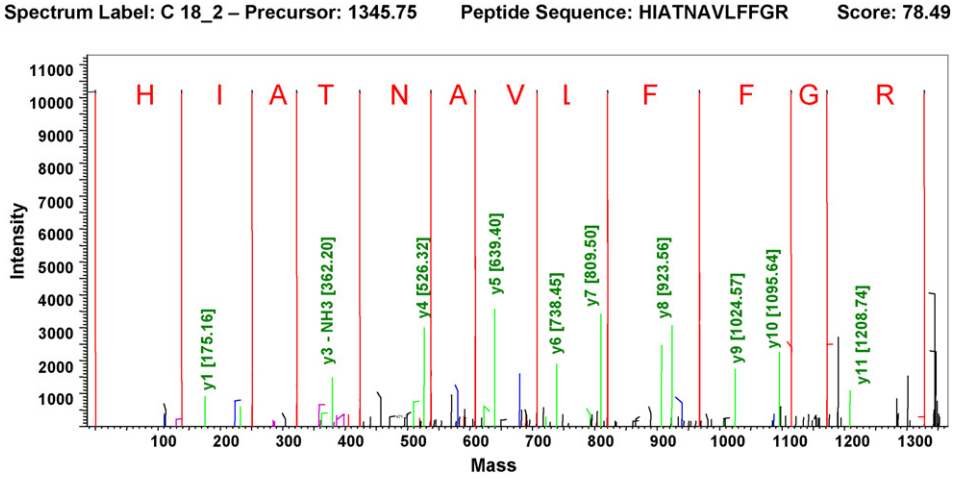

Fig. 7. Sequence spectrum of isolated allergenic epitope of ovalbumin (*m*/*z* 1345.75) obtained by tandem MS sequencing on Q-TOF, fragment ions (y-series) are labeled (determinated sequence: HIATNAVLFFGR, score: 78.49).

ments 41–172 and 301–385 in two-dimensional (2D) PAGE blots suggesting that these fragments are involved in an immunogenic structure.

Observed differences in length and localization of epitopes of interest in some published studies may be attributed to: (1) difference in the patient populations employed in each study (patients with persistent or outgrown allergy [31], genetic variability of individuals can also generate a diversity of recognized epitopes and influence their hierarchy), (2) the way of entry rout [34], (3) different conformations of ovalbumin [38] (intact tertiary structure or partially or totally digested form), (4) type of utilized proteolytic enzyme (various peptide fragments can be obtained by digestion with different enzymes) and (5) type of utilized specific antibodies (source, clonality and isotype can influence specific reactivity and affinity). Such information contributes greatly not only to the elucidation on the etiology of allergy, but also to the development of strategies for design of peptide-based vaccines for specific immunotherapy.

Our strategy, combined effective and contaminant-free digestion step with immunoaffinity capturing situated in microfluidic device, provides rapid and simple way how to identify the epitopes of interest. Due to the minimal demands on the biological material and reagents, the costs are acceptable for routine testing. Taking the specific anti-ovalbumin antibodies of patients' serum we can identify the main allergenic structure specific for the individuals and get the information for preparation of peptide-based autovaccine.

We believe that the work described here is broadly applicable and provides a flexible and efficient analytical approach for screening of allergens, autoantigens and other immunogenic biomolecules.

4. Conclusion

A microfluidic device with an integrated bioanalytical system has been tested for the structural analysis of the food allergen ovalbumin. The major goal of this study, to develop and verify the new methodological strategy for rapid and cost-effective identification of the significant immunogenic epitopes, has been successfully achieved. In agreement with already published studies, the tryptic fragment HIATNAVLFFGR was identified as an allergenic epitope of ovalbumin. The benefits provided by a combination of magnetic bead-based digestion and immunocapturing with a microfluidic analytical approach were confirmed. This novel analytical concept, chip-based epitope extraction technique, offers many advantages and can be an appropriate tool for the rapid and simple structural characterization of antigens and for design of risk-free immunotherapy in general.

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