Chapter 7
Affinity-Mass Spectrometry Approaches for Elucidating Structures and Interactions of Protein–Ligand Complexes

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Abstract Affinity-based approaches in combination with mass spectrometry for molecular structure identification in biological complexes such as protein–protein, and protein–carbohydrate complexes have become popular in recent years. Affinity-mass spectrometry involves immobilization of a biomolecule on a chemically activated support, affinity binding of ligand(s), dissociation of the complex, and mass spectrometric analysis of the bound fraction. In this chapter the affinity-mass spectrometric methodologies will be presented for (1) identification of the epitope structures in the Abeta amyloid peptide, (2) identification of oxidative modifications in proteins such as nitration of tyrosine, (3) determination of carbohydrate recognition domains, and as (4) development of a biosensor chip-based mass spectrometric system for concomitant quantification and identification of protein–ligand complexes.

Abbreviations

ACN	Acetonitrile
AD	Alzheimer's disease
APP	Amyloid precursor protein
CE	Equivalent carbon
CRDs	Carbohydrate recognition domains

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CREDEX	*C*arbohydrate *re*cognition *d*omain *ex*cision
2-DE	Two-dimensional electrophoresis
DHB	2,4-Dihydroxybenzoic acid
ECP	Eosinophil cationic protein
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FTICR	Fourier transform ion cyclotron resonance
HCCA	α-Cyano-4-hydroxy-cinnamic acid
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
3NT	3 Nitrotyrosine
PROFINEX	*Pro*teolytic af*fin*ity *ex*traction
SAS	Solvent accessible surface
SAW	Surface acoustic wave
TFA	Trifluoroacetic acid
ToF	Time of flight

7.1 Introduction

Proteins have the ability to specifically interact with one or more ligands. Protein–ligand complexes such as protein–protein, protein–carbohydrates, and protein–DNA play an essential role in a multitude of physiological and pathophysiological cellular processes. Determination of protein–ligand-binding affinity is crucial for the mechanistic understanding of protein's function and for the development of new biochemical, analytical, or biomedical applications.

Traditionally, the structure of proteins in complex with their ligand(s) have been determined by X-ray crystallography, nuclear magnetic resonance (NMR), fluorescence and IR spectroscopy, and surface plasmon resonance [11, 26, 31, 52]. However, these methods have a number of limitations such as: (1) they require relatively large amounts of pure protein, (2) many of these studies were performed under nonequilibrium conditions, and (3) they are time consuming, through elaborate and lengthy experimental protocols [52]. Also, the specificities of protein/peptide antigen–antibody interactions were used either to obtain purified proteins from complex mixtures (such as serum or cell lysates) or for antibody purification. The former is typically performed using monoclonal or polyclonal antibodies attached covalently to resins [49], while the latter is based on immunoglobulin-specific protein A or protein G affinity columns [10]. Immunoaffinity techniques such as Western blot [15, 21], immunohistochemistry [45], and ELISA [17, 23] rely on the use of antibodies of various specificities to detect antigen–antibody interactions, providing information about the overall binding and strength of the interaction. However, no details about the chemical structures of antibody–antigen binding can be determined.

Mass spectrometric methods can provide structural details on many levels for different classes of biomolecules; proteins can now be analyzed by mass spectrometry to reveal complete or partial amino acid sequences, posttranslational modifications, protein–protein interaction structure sites, and even provide insight into higher order structure [44, 55]. One key advantage of mass spectrometric measurements is its capability to analyze extremely small quantities of sample with high sensitivity. The "soft" ionization/desorption techniques such as electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI) were major tools used to developed affinity-mass spectrometric methods that provide key data to identify the specific interacting structures within protein–ligand interactions.

The combination of affinity-mass spectrometry (affinity-MS) was shown in the last years, as an established methodology for (1) direct protein identification from complex biological material [22], (2) identifications of epitope [9, 43, 46, 56], paratope [42], and peptibody [41, 54], and (3) the identification of carbohydrate recognition domains (CRDs) [29]. These results were possible by developing and applying new approaches of affinity-MS in combination with selective proteolytic digestion (epitope excision) and affinity selection of proteolytic generated fragments (epitope extraction) as shown in Fig. 7.1. The development of these two affinity approaches were based on the hypothesis that (1) in epitope excision, an antibody will protect the binding site(s) of a bound protein or peptide antigen from proteolytic cleavage, and (2) in epitope extraction, an antibody will bind a minimum epitope sequence generated by the proteolytic cleavage of the antigen molecule in solution. The epitope excision (Fig. 7.1a) consists in immobilizing the antibody on a Sepharose material in a micro-column and binding the antigen peptide or protein to this column. Then, a specific protease is added to the column, and the proteolytic digestion is performed. The non-bound fragments are washed away, and the epitope–antibody complex is dissociated by using acidic condition (elution). In the epitope extraction (Fig. 7.1b), the antigen molecule is first proteolytically digested in solution, and the resulted fragments are added on the antibody column and allowed to bind. Again, the non-bound fragments are washed away, and the epitope–antibody complex is eluted by using acidic condition. The last washing fraction and elution fraction are analyzed by mass spectrometry for the identification of epitope structure.

The direct, chemical epitope identification approach using epitope excision/extraction-mass spectrometry provided differential information about the epitope structures by comparison of shielding of specific proteolytic cleavage sites comprised in the epitope peptide; information that cannot be directly determined by affinity techniques such as Western blot, quantitative ELISA, and SPR. However, the epitope identity can be readily ascertained and verified by affinity studies using synthetic mutated peptides in affinity-mass spectrometric approaches.

This chapter describes recent progress made in the area of affinity-mass spectrometric-based approaches for (1) identification of epitope structures in Abeta amyloid peptide, (2) specific identification of oxidative modifications in proteins, (3) for determination of CRDs, and as (4) development of a biosensor chip-based system.

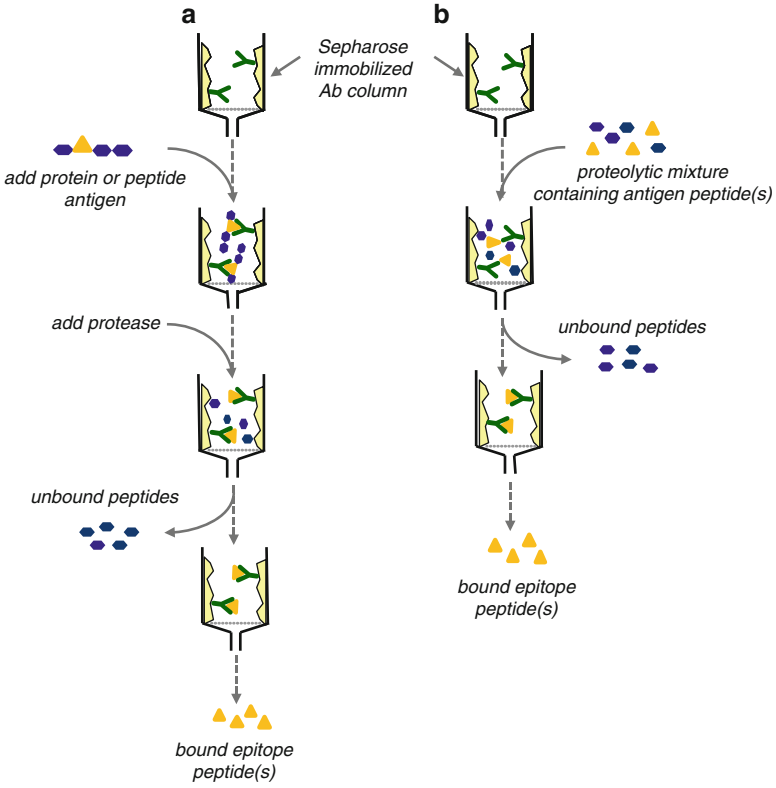

Fig. 7.1 Analytical scheme of affinity-mass spectrometric identification of antigen epitope(s). (**a**) In *epitope excision* the antigen is bound to the immobilized antibody column, and then proteolytical cleavage is performed using protease and (**b**) in *epitope extraction* the antigen is first degraded proteolytically in solution and then applied on to the immobilized antibody column. The resulted epitope containing fractions are analyzed by mass spectrometry

7.2 Materials and Methods

7.2.1 Immobilization of Antibodies to NHS-Activated Sepharose

For preparing the affinity column, an aliquot of 100 µg anti-Aβ(1-42) polyclonal antibody from TgCRND8 mouse or anti-3nitrotyrosine (3NT) monoclonal antibody (1 µg/µL) was mixed with coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and added to the appropriate amount of dry NHS-activated 6-aminohexanoic acid-coupled Sepharose (1 g Sepharose swelled in approximately 3 mL of coupling buffer). The coupling reaction was performed for 2 h at 25 °C under vigorous shaking. The reaction mixture was then loaded into a micro-column (MoBiTec, Göttingen, Germany) and extensively washed by alternating twice 10 mL blocking buffer

(0.1 M ethanolamine, 0.5 NaCl, pH 8.3), with 10 mL washing buffer (0.2 M NaOAc, 0.5 M NaCl, pH = 4.0). To block unreacted active groups, the affinity matrix was kept in blocking buffer for 1 h at room temperature; afterwards, the washing step was performed using 30 mL PBS buffer, pH = 7.4. The affinity column was stored in PBS buffer at 4 °C.

7.2.2 *Identification of the Abeta (1-40) Epitope by Proteolytic Epitope Excision/Extraction-Mass Spectrometry*

For *epitope excision* experiments 100 μg of antigen Aß(1-42) synthetic peptide were applied onto the immobilized antibody column. The micro-column was then gently shaken for 1 h to allow complete binding of antigen. The column was then washed with 20 mL PBS buffer for removal of unbound antigen peptide, and the remaining affinity-bound Aß(1-40) peptide was digested for 2 h at 37 °C by addition of 0.2 μg of protease (trypsin, or α-chymotrypsin and aminopeptidase M) in 200 μL PBS buffer. Supernatant non-epitope fragments were removed by washing with 20 mL PBS buffer. After removal of the unbound peptide fragments, the immune complex was dissociated from the immobilized antibody by addition of 500 μL 0.1 % TFA; the column was shaken gently for 15 min, and the released epitope peptides were collected into a micro-centrifuge tube. The samples were then lyophilized and stored until mass spectrometric analysis. The column was regenerated by washing with 10 mL 0.1 % TFA followed by 20 mL PBS buffer. When handled in this way, affinity micro-columns may be used at least 10–20 times without significant loss of antigen-binding capacity. With the immobilization and proteolytic digestion conditions employed, IgG antibodies generally are highly stable towards degradation, as established in previous studies [33, 43].

For the *epitope extraction* experiments, the antigen, Aß(1-40) synthetic peptide, was first digested with the chosen protease. After 2 h digestion time at 37 °C the resulted peptide mixture was added to the immobilized antibody column. The binding of the antigen peptide fragment to the antibody was performed for 1 h at RT. The antigen peptide retained on the column was eluted with 500 μL 0.1 % TFA. In all experiments the supernatant, the final milliliter of the washing fraction, and the elution fraction were lyophilized, desalted by Zip-Tip®, and analyzed by mass spectrometry.

7.2.3 *Proteolytic Affinity Extraction-Mass Spectrometry for Identification of Tyrosine Nitration Sites in Human Eosinophil Proteins*

For the proteolytic affinity peptide extraction, highly pure (>90 %) human eosinophil cationic protein (ECP) was denatured and digested in solution. The protein was dissolved first at a concentration of 1 μg/μL in 10 mM NH_4HCO_3 (pH 8) and then DTT in 10 mM NH_4HCO_3 (50-fold excess, relative to the number of

–S–S-bound in protein) was added. The reduction was carried out for 1 h at 56 °C under gentle shaking. After cooling to room temperature, a solution of IAA in MilliQ was added in 2.2-fold excess relating to DTT amount, and the reaction was performed for 45 min at ambient temperature in the dark with occasional vortexing. After lyophilization ECP was proteolytically digested by trypsin in 10 mM NH_4HCO_3, pH = 8. Trypsin was added to the sample at an enzyme to substrate ratio of 1:30 (w/w), and the digestion was carried out at 37 °C for 4 h and then quenched by freezing the sample with liquid nitrogen. The resulting peptide mixtures were analyzed directly by mass spectrometry or were used for immunoaffinity experiments. The resulted ECP peptides fragments were added onto the anti-3-NT-antibody column and incubated under gentle shaking for 2 h at room temperature. The non-bound peptides fragments (supernatant fraction) were removed by pushing air through the column using a 10 mL syringe. The matrix was subsequently washed with 200 mL PBS buffer for removal of unbound peptides. The immune complex between nitrated peptide and anti-3-NT antibody was dissociated by addition of 0.1 % TFA. The collected fractions were analyzed by MALDI-TOF and nano-ESI FT-ICR-MS. In a second experiment the elution fraction was analyzed by Edman sequencing.

7.2.4 Online SAW Biosensor–ESI-MS Coupling

A Bruker Esquire 3,000+ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) was coupled online with an S-Sens K5 biosensor (Biosensor GmbH, Bonn, Germany) via an manual interface. The interface developed for online coupling of surface acoustic wave (SAW) biosensor with an ESI-ion trap mass spectrometer utilizes a six-port valve micro-column and micro-injector for desalting and in situ concentration of protein samples dissociated from the protein–ligand complex as illustrated in Fig. 7.6. The C_{18}-reversed phase guard column coupled to the microinjector unit was from Rheodyne (California, USA). The antibody was immobilized on carboxyl self-assembled monolayer (SAM) surface via available amide bond (N-terminal or lysine residues). SAM is formed first by activation of the carboxyl group with 200 mM *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and second by coupling reaction using 50 mM *N*-hydroxysuccinimide (NHS). A solution of 300 nM of antibody was immobilized on the preactivated-SAM surface, followed with capping of non-reacted NHS groups with 1 M ethanolamine pH 8.5. A 10 μM solution of peptide ligands mixture was added, and affinity binding was performed and recorded at a flow rate of 20 μL/min. All affinity binding experiments were performed at 20 °C in PBS binding buffer, pH 7.5. Following association of ligand(s), elution was carried out with glycine buffer, pH 2, cleaning of buffer salts and transfer of the eluted compound into the ESI source were performed using 0.3 % aqueous HCOOH (desalting step; approx. 300 μL at 70 μL/min) and with 0.3 % HCOOH/80 % acetonitrile (elution step; 70 μL/min), respectively, by switching of the microvalve injector. Determinations of dissociation constants were typically performed after

immobilization of peptide using a 10 μM solution on the activated SAM surface at a flow rate of 20 μL/min. The flow was then changed to PBS buffer, pH 7.5, and a solution of 350 nM anti-lysozyme antibody was added to block all unspecific binding sites, followed by regeneration with 150 μL glycine buffer. Association kinetics were determined with serial dilutions of 8–128 nM of antibody, followed with regeneration by glycine buffer, pH 2, and concentrations and volumes of ligands were optimized to obtain highest binding curves. Determination of antibody/protein association kinetics was performed by extracting the data from the sensor signals for all concentrations employed, using the 1:1 monomolecular growth model. Determination of the dissociation constant is provided after plotting the pseudo-first kinetic constant versus concentration, and applying a linear regression by $Kd = koff \times kon^{-1}$.

7.2.5 *Proteolytic Excision/Extraction-Mass Spectrometry of Ligand-Binding Peptides in Human Galectins-1 and Galectins-3*

The covalent coupling of lactose to divinylsulfone-activated Sepharose 4B was performed as previously described [6]. For proteolytic CRDs' *peptide excision*, a solution of 50 μg human galectin-1 or galectin-3 in 100 μL 50 mM PBS buffer, pH 7.5, was added to 200 μL affinity matrix and allowed to bind at 37 °C for 24 h. Any remaining unbound galectin was washed out with 30 mL binding buffer, and the washing fractions were collected and analyzed by MALDI-TOF-MS in order to determine the extent of binding. The digestion of bound galectin was performed using trypsin, in the binding buffer, with an enzyme to substrate ratio of 1:100, at 37 °C, for 3 h. After digestion the supernatant was analyzed by MALDI-FTICR-MS. The free tryptic peptides (not bound to the carbohydrate) were washed out with 30 mL PBS buffer, pH 7.5. The washing fractions were analyzed by MALDI-FTICR-MS. The affinity-bound peptides were eluted under strong shaking with 400 μL 0.3 M lactose in PBS buffer, pH 7.5, at 37 °C for 15 min, and this procedure was repeated twice. For galectin-1 protection of Cys residues from oxidation was performed using 0.6 mg DTT in the binding buffer and 1 g/L DTT in the washing buffer.

In a second experimental approach, proteolytic CRDs *peptide extraction*, 50 μg human galectin-1 or galectin-3 was digested in solution using trypsin (enzyme: substrate ratio, 1:20) for 5 h at 37 °C in 200 μL PBS buffer, 50 mM at pH 7.5. A sample aliquot from the digestion mixture was analyzed by MALDI-MS. The rest of the digest was added over an affinity column containing 200 μL matrix. After incubation for 12 h at 37 °C, the supernatant was removed and analyzed by MALDI-MS. Any traces of free peptides were washed out from the column with 10 mL PBS buffer, pH 7.5, and the washing fraction was analyzed by MALDI-FTICR-MS. The peptides bound to lactose were eluted under shaking with 400 μL ACN: 0.1 % TFA 2:1 at 37 °C for 15 min, repeating the procedure twice.

7.2.6 *Mass Spectrometry*

MALDI-ToF-MS was performed with a Bruker Biflex™ linear TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen UV laser (337 nm) and a dual channel plate detector. A saturated solution of α-cyano-4-hydroxy-cinnamic acid (HCCA) in ACN: 0.1 % TFA (2:1) was used as matrix. Acquisition of spectra was carried out at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV.

High-resolution FTICR-MS was performed with a Bruker Daltonics Apex II instrument equipped with a 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell, and a Scout-100 MALDI or a nano-ESI ion source. A 50 mg/mL solution of 2,4-dihydroxybenzoic acid (DHB) in ACN: 0.1 % TFA (2:1) was used as matrix for MALDI-FTICR MS. Nano-ESI-FTICR mass spectra were obtained by accumulation of 15 single scans, with the capillary exit voltage set to 20 V and the skimmer 1 set to 10 V, while the capillary voltage was adjusted between −1,100 and −1,200 V until a stable spray was obtained. The ions were accumulated in the RF-only hexapole for 0.15 s before being transferred into the ICR cell. Calibration, acquisition, and processing of spectra were carried out with the Bruker XMASS Software.

7.3 Results and Discussions

7.3.1 *Affinity-Mass Spectrometric Approaches for Elucidation of a β-Amyloid-Plaque-Specific Epitope*

Amyloid-ß-peptide (Aß) fragment(s) of the amyloid precursor protein (APP) is the major peptide(s) constituent of senile amyloid plaques in brains, which represent hallmark of Alzheimer's disease (AD). The formation of neurotoxic oligomeric soluble intermediates precedes aggregation and is critical to overall neurotoxicity and progressive neurodegeneration [30]. Although Aß has been studied thoroughly, molecular details of pathophysiological degradation, chemical structure modification, Aß aggregation mechanism, and its cellular interactions remain unclear [2, 20]. Understanding pathophysiological amyloid-ß-peptide aggregation and its interactions with ligands such as antibodies or protective proteins is a key prerequisite for the development of drugs capable of (1) neutralizing or disaggregating amyloid-ß aggregates or (2) inhibiting the initial step(s) of aggregate formation.

Highly efficient affinity-MS methods such as proteolytic epitope excision and proteolytic epitope extraction have been developed and applied to elucidate the molecular mechanism of immunotherapeutic approaches involving Aß "plaque-specific" antibodies. These are produced in transgenic AD mouse models upon active immunization with Aß(1-42) and were found to promote disaggregation of Aß plaques and fibrils and to improve the memory impairment in AD mice [25, 27].

Epitope excision/extraction experiments were performed using the immobilized polyclonal antibody from TgCRND8 mouse (see Sect. 2.1) [14]. The antigen peptide Aß(1-40) was bound to the antibody column; the immuno-Aß(1-40) complex was allowed to form, and then trypsin was added onto the column as described in Sect. 2.2. After removal of the unbound fragments generated by proteolysis, the immune complex was dissociated by addition of 0.1 % TFA. In the MALDI-FTICR mass spectrum (Fig. 7.2) of the supernatant fraction (A), all expected tryptic fragments of ß-amyloid Aß(1-40) peptide were present. After washing off the unbound fragments, in the spectrum of the elution fraction (B), only a single peak corresponding to the Aß peptide [1-16] fragment was identified. This fragment was the only one which remained bound to the polyclonal antibody and proved that the epitope recognized by the polyclonal antibody from TgCRND8 transgenic mouse was in this N-terminal region of the peptide.

For epitope extraction, the Aß(1-40) was first digested using trypsin, and the tryptic peptide mixture was added on the same antibody column upon regeneration. The extraction experiment led to similar results, as the Aß(1-16) tryptic peptide was identified in the elution fraction. Therefore, further experiments were performed in order to determine in detail the minimum structure of the Aß(1-40) epitope.

A second epitope excision experiment with Aß(1-40) was carried out as described before but using endoprotease GluC instead of trypsin. This protease was chosen because it cleaves the peptide bonds after glutamic (E) and aspartic (D) acid residues, enabling determination of shorter peptide sequences if these still remain bound to the antibody. Analyzing the elution fractions by high-resolution MALDI-FTICR MS, only a single peptide fragment corresponding to the amino acid sequence Aβ (4-FRHDSGYE-11) was found bound to the polyclonal antibody column. A more detailed epitope elucidation experiment was carried out using a combination of α-chymotrypsin and aminopeptidase M. Amino peptidase M is an exopeptidase that cleaves all amino acids starting from the N-terminal part of the antigen sequence. For the epitope excision experiments, the antigen peptide, Aß(1-40), was bound to the polyclonal anti-Aß(1-42) antibody column, digested with α-chymotrypsin and afterwards with aminopeptidase M with washing steps in between. The MALDI-FTICR mass spectra of the supernatant and elution fractions are shown in Fig. 7.3. α-Chymotrypsin digestion of Aß(1-40) has generated the fragment (1-10), which was further digested by aminopeptidase M.

From the MALDI-FTICR mass spectrum of the elution fraction, the smallest fragment bound to the polyclonal anti-Aß(1-42) antibody column was Aß(4-10) peptide. This represents the minimal structure of the ß-amyloid peptide recognized by the polyclonal antibody. The specific N-terminal amino acid sequence Aß (4-FRHDSGY-10) was identified with high mass accuracy as the plaque-specific epitope, providing a basis for the development of new lead structures for AD vaccine development [39, 42]. The identification and quantification of Aß peptides bound to specific anti-Aß antibodies were provided by the newly introduced online bioaffinity-electrospray mass spectrometry [5]. In several further studies, proteolytic excision/extraction-mass spectrometry were successfully used for the identification of interacting Aß epitope sequences with aggregation-inhibiting polypeptides

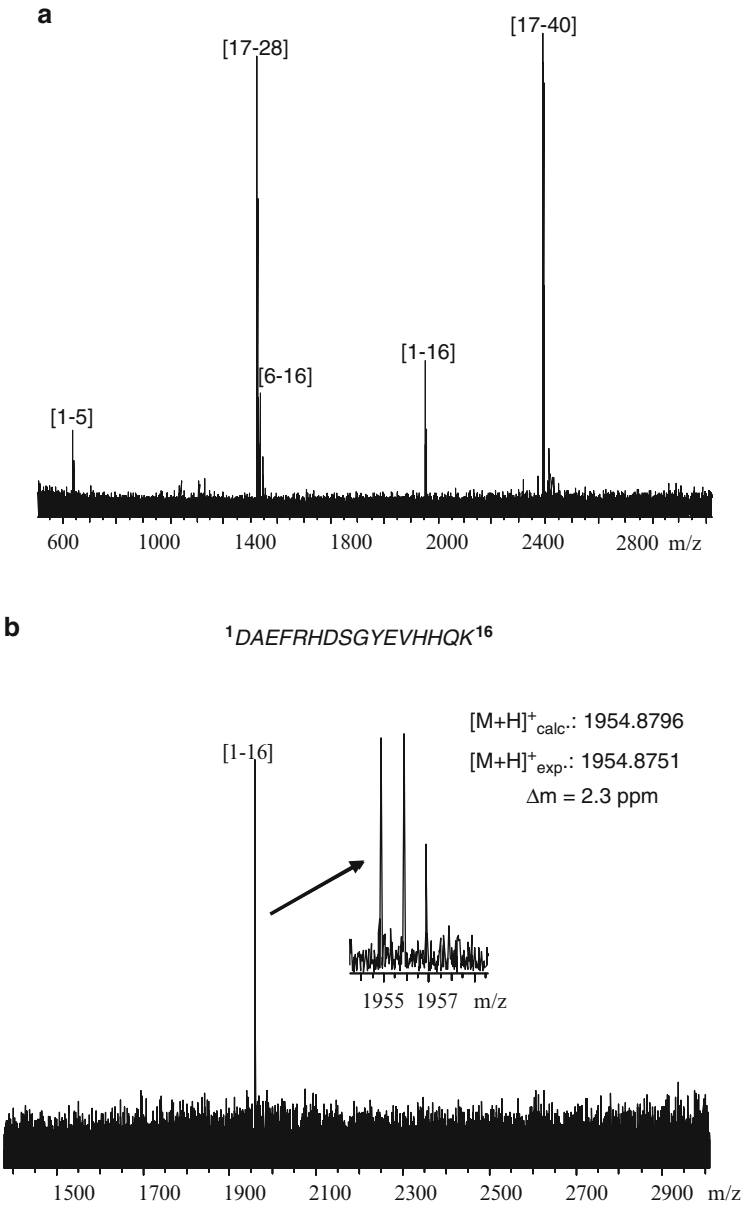

Fig. 7.2 Mass spectrometric epitope excision of Aß(1-40): (**a**) MALDI-FTICR MS of the supernatant fraction of Aß(1-40) after trypsin digestion covering the whole peptide sequence. (**b**) MALDI-FTICR MS of the elution fraction reveals only one Aß peptide fragment (1-16). Reprinted with author permission from [14]

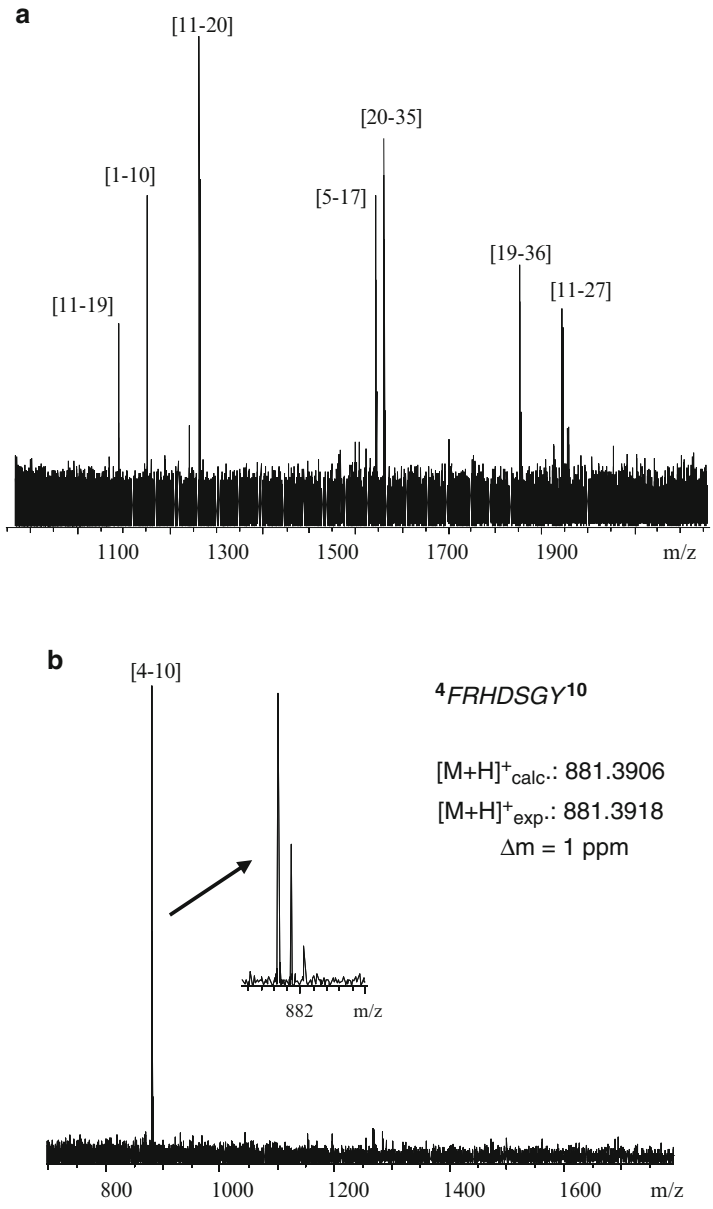

Fig. 7.3 Mass spectrometric epitope excision of Aß(1-40) using α-chymotrypsin and aminopeptidase M. (**a**) MALDI-FTICR MS of the supernatant fraction and (**b**) MALDI-FTICR MS of the elution fraction reveal minimum peptide fragment Aß (4-10) which remains bound to the antibody column. Reprinted with author permission from [14]

such as cystatin C [16], humanin [24], and Aß-nanobodies (single chain llama anti-ß-amyloid antibodies) providing also a potential for developing new diagnostic tools for AD as well as for designing new potential immunotherapeutic agents [34].

7.3.2 *Proteolytic Affinity Extraction-Mass Spectrometric Approach for Identification of Tyrosine Nitration Sites*

Tyrosine nitration represents an oxidative modification in proteins. It has been shown to occur under physiologic conditions, but found to be substantially enhanced under various pathophysiological conditions such as atherosclerosis, asthma and lung diseases, neurodegenerative diseases, and diabetes [2, 7]. For the molecular correlation of protein nitration with pathogenic mechanisms of human diseases and with animal or cellular models of diseases, it is essential to identify the protein targets of nitration and the individual modification sites. Most identifications of protein nitrations have been obtained using immunoanalytical methods such as Western blot, ELISA, and immuno-electron microscopy, employing different 3-NT-antibodies. These antibodies were poorly characterized regarding their specificities, providing only the overall detection of nitrations, and no identification of specific nitration sites and structures [12, 13]. A combination of electrophoresis, Western blotting, and mass spectrometry has been applied in several proteomics studies of nitrated proteins from biological material [1, 2, 8]. However, specific 3-nitrotyrosine modification sites were not identified, rendering the identification of nitration sites critically dependent on the specificity of NT-antibodies. The failure of many mass spectrometric-based approaches to characterize nitrated proteins may be due to multiple causes such as (1) low abundance of NT-containing proteins/peptides, (2) solubility problems, hydrophobicity, and/or extreme pI values of proteins, which may compromise the isoelectric-focusing in 2-DE separations, (3) insufficient recovery of NT-containing peptides from gels and/or HPLC columns during LC-MS analysis, and (4) instability of nitrated peptides due to photochemical decomposition of nitro-tyrosine residues under UV-MALDI-MS analysis condition [1, 2, 38]. The problems of identification of tyrosine nitration at biologically relevant levels in nitrated proteins have been overcome by the recent development of a proteolytic affinity extraction-MS "PROFINEX" [37], analogue to epitope extraction procedure described above (Sects. 2.2 and 3.1). The efficiency of the PROFINEX approach is shown here in the example of human ECP isolated from patients with abnormal elevate number of eosinophils in blood [47]. First ECP was denatured and digested in solution, and the resulted tryptic peptide mixture was added to the immobilized 3NT-antibody column. The 3NT-antibody–ECP peptide(s) complex was allowed to form, and unbound ECP tryptic peptides were removed by washing. Due to the high specificity of the 3NT-antibody for the antigen, only peptides containing the antigenic determinant (the 3-nitro-tyrosine) should interact with the antibody. Antibody-bound ECP peptide was eluted by dissociating the immune complex under slightly acidic condition (pH = 2.5). The supernatant, wash, (last mL of wash) and elution fractions were collected, lyophilized, and analyzed by mass

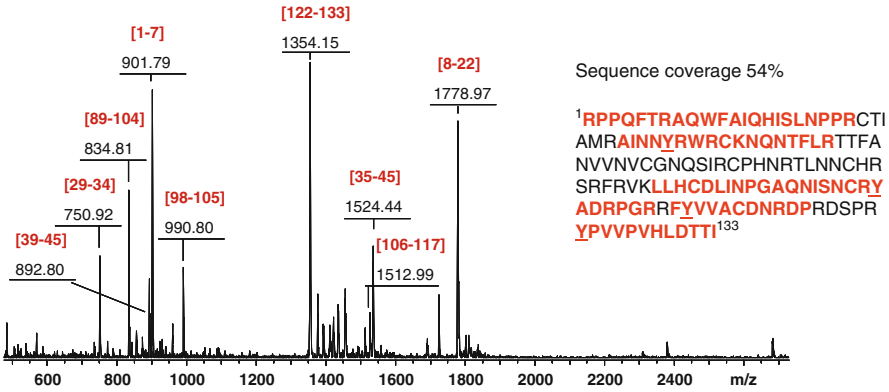

Fig. 7.4 Proteolytic affinity extraction-mass spectrometric approach. MALDI-TOF mass spectrum of unbound ECP tryptic peptides contained in the supernatant fraction. All four tyrosine residues in eosinophil cationic protein were found to be unmodified

spectrometry after desalting by C_{18} ZipTip. The unbound tryptic peptide contained in the supernatant fraction was analyzed by MALDI-TOF (Fig. 7.4). All four tyrosine residues contained in ECP were found unmodified. The identified peptides accounted for 54 % sequence coverage in the supernatant fraction.

The unequivocal identification of the peptide containing nitrated Tyr was obtained by nano-ESI-FT-ICR analysis of the eluate (Fig. 7.5). The spectrum contained a doubly protonated molecular ion at *m/z* 764.3618, corresponding to the ECP peptide ^{23}CTIAMRAINNY(NO_2)R^{34}, and nitrated at Tyr^{33} residue. The monoisotopic mass of the singly charged molecular ion was determined after deconvolution of the ESI mass spectrum using the XMASS software and was assigned to ECP peptide fragment (23-34) with a mass difference of 103 Da (45 Da for the nitro group and 58 Da for Cys^{23}—carbamidomethyl formation).

The missed cleavage at Arg^{28} indicates that this residue is part of an "epitope" structure within ECP only upon nitration of Tyr^{33}, because the same arginine residue was cleaved by trypsin in the non-nitrated Tyr^{33} containing peptide (Fig. 7.4). A spatial orientation of tyrosine residues in ECP was illustrated using the crystal structure available in the UniProt database (PBD accession number 1H1H) [28]. Using the molecular modeling software BallView 1.1.1, the secondary ribbon structure was rendered and is shown in Fig. 7.5. The location of Tyr^{33} within a loop structure is indicated by yellow, and all other three tyrosine residues were colored. The solvent accessible surface (SAS) defined by using this molecular modeling program showed that Tyr^{33} residue of ECP has the highest surface accessibility (94.4 Å^2), while the other three Tyr residues are embedded in the tertiary structure of the molecule. Moreover, the hydroxyl group of Tyr^{33} and the two equivalent *ortho* carbons CE_1 and CE_2 extremely protrude in the exterior space. These are critical for allowing tyrosine to be modified by nitration. The molecular SAS model shows that Tyr^{98} is completely buried in the protein core, and its hydroxyl group is not exposed to the surface, while for Try^{107} and Try^{122} the hydroxyl groups and the nearby carbons appear less surface exposed [35].

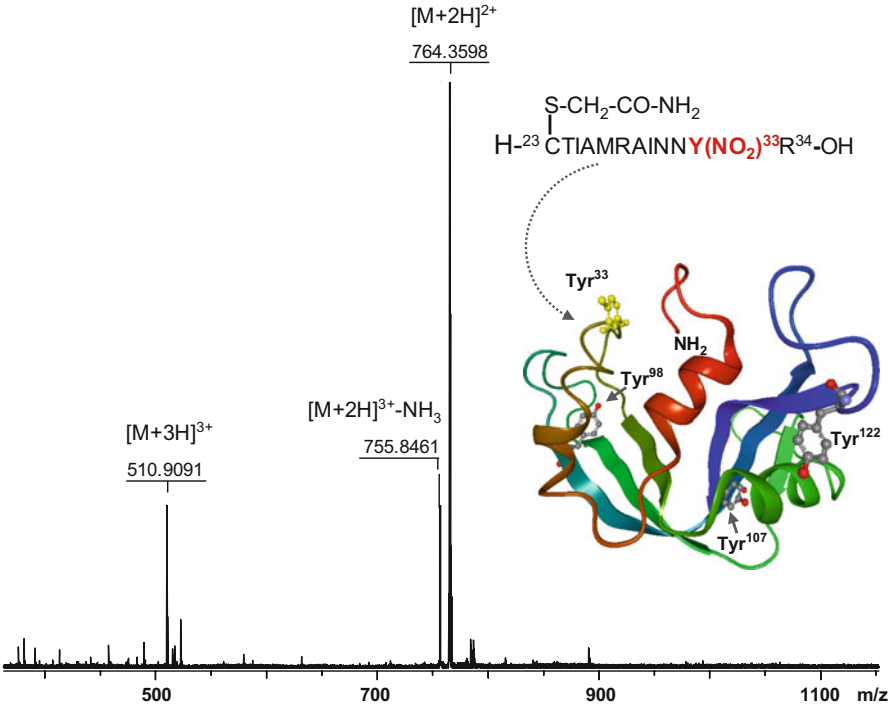

Fig. 7.5 Proteolytic affinity extraction-mass spectrometric approach. Nano-ESI FTICR mass spectrum of the elution fraction containing the nitrated ECP (23-34) peptide fragment, with nitro-Tyr^{33} residue. The inset shows the modeling structure of ECP indicating the accessible surface area of the protein rendered using BallView 1.1.1 program based on the available X-ray crystal structure of the ECP (PDB entry 1H1H)

Furthermore, the combination of proteolytic affinity peptide extraction and Edman sequencing provided quantitative information about the endogenous nitration of ECP in human eosinophil granules. Proteolytic affinity peptide extraction was performed for ECP protein as described above followed by analyzing the elution fraction by Edman sequencing. The quantification was obtained by determination of initial and repetitive yields in each sequencing cycle. From the ratios of sequenced nitrated peptides to intact ECP protein used for PROFINEX, approximately 15 % nitration level was determined for native nitrated ECP [35].

7.3.3 Online Bioaffinity-Mass Spectrometry for Molecular Recognition Specificity of Anti-3-nitrotyrosine Antibodies

Another recently developed analytical tool for measuring molecular interaction kinetics and identifying binding-specific structures is the online combination of SAW biosensor with electrospray ionization MS (SAW-ESI-MS). SAW technology uses the piezoelectric effect of amount differences loaded on a chip to detect bioaffinities with high sensitivity in dilute solutions, without labeling approaches or recalibration for

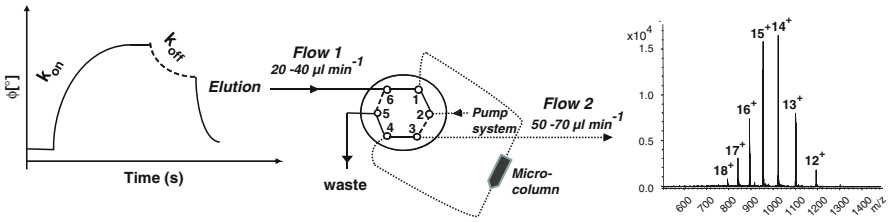

Fig. 7.6 Schematic representation of online combination of SAW biosensor instrument with ESI-MS using a six-port valve micro-column interface. The waste capillary from the SAW biosensor is connected to the interface in position 6 and the ESI-MS inlet capillary in position 3. Total dissociation of protein(s) from the antibody surface was performed by elution with acidic buffer to the micro-column interface (flow 1). This was followed by cleaning with washing solution, elution with aqueous acetonitrile/HCOOH, and transfer of the eluate into the ESI source by switching the injector position (flow 2). Adapted with kind permission of authors and printed with kind permission of Springer Science and Business Media from J. Am. Soc. Mass Spectrom. 21(10): 1643-8, Drăguşanu M., et al. (2010)

buffer changes [18, 40]. One of the first experiment we performed by SAW-ESI-MS online coupling enabled the direct detection, identification, and quantification of nitrated model peptides' affinities to a 3 NT-specific antibody [5]. A six-port valve inject unit system was used between the SAW biosensor and the ESI mass spectrometer equipped with a micro-guard column; the combination provides simultaneous sample concentration, desalting, and flow rate adjustment for mass spectrometric analysis of the dissociated ligand. The interface used for online coupling of SAW biosensor with an electrospray ion trap mass spectrometer is illustrated in Fig. 7.6 and described in detail by Dragusanu et al. [5]. After SAW biosensor measurement of the peptide–antibody association, the peptide is eluted into the guard column (Fig. 7.6, flow 1) and washed to remove PBS buffer salts. An HPLC-pumping system was used for performing the elution of the peptide from the guard column into a manual-assisted microvalve injector which further transferred the sample into the ESI source (Fig. 7.6, flow 2). This interface system provided routine mass spectrometric analysis of real time peptide/protein association from the biosensor surface and showed long-term operation stability with minimal contamination of the ESI-MS system [5].

As shown before (Sect. 3.2) the use of anti-3-nitro-tyrosine-specific antibodies has been essential for mass spectrometric identification of tyrosine nitrations using proteolytic affinity-MS. Poor information regarding the specificities of anti-3-nitrotyrosine antibodies has been reported before [4]. In a previous publication we described a molecular study of the recognition specificities and affinities of two commercially available, monoclonal anti-nitrotyrosine antibodies by dot-blot, ELISA, and affinity-MS, using different 3-nitrotyrosine-containing peptides [36]. The comparison of the three methods essentially provided consistent results to reveal differences in binding affinities and specificities by the anti-nitrotyrosine antibodies, suggesting that antibody binding may be influenced by the peptide structure adjacent to the nitrotyrosine modification.

Several nitrated peptides from ECP were synthesized by solid-phase peptide synthesis, purified by reversed phase-HPLC and characterized by mass spectrometry.

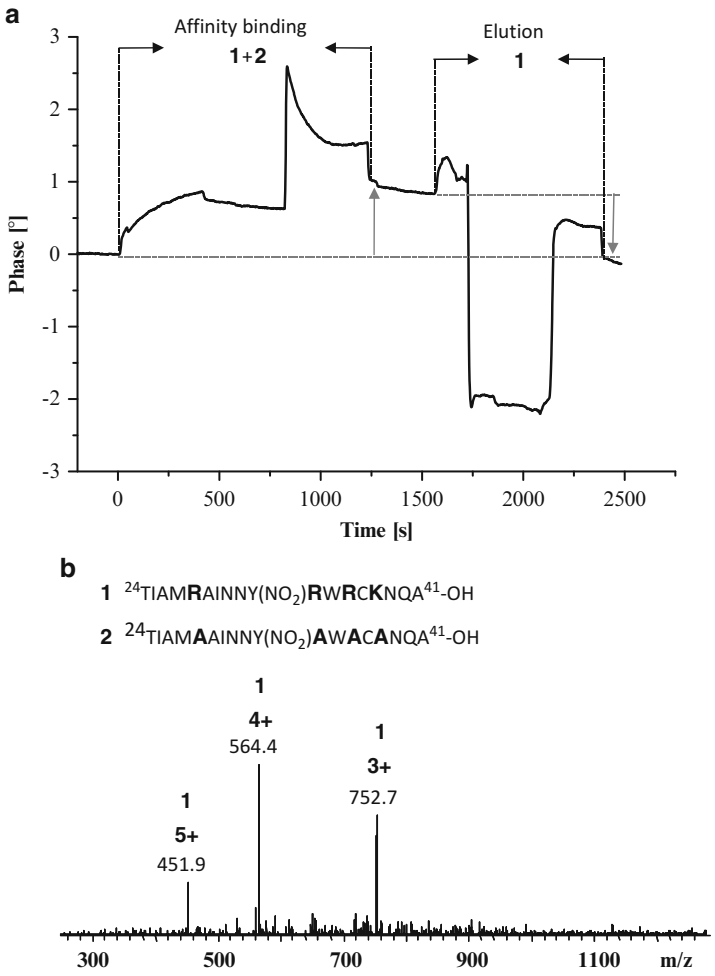

Fig. 7.7 (**a**) SAW binding and elution curves of the peptides mixture using online SAW-ESI-MS and (**b**) ESI-MS spectra of the eluted fraction illustrating the mass spectrometric identification of Tyr-nitrated ECP peptide **1** bound to the immobilized 3-NT antibody. Reprinted with kind permission of authors and Springer Science and Business Media from J. Am. Soc. Mass Spectrom. 23(11): 1831-1840, Petre B.A., et al. (2012)

These model-nitrated peptides have been used to study their affinity to monoclonal anti-3-NT antibodies by online-SAW-ESI-MS. For example, the immobilization of an anti-3NT antibody on the biosensor chip surface via a SAM is performed as a first step. Following association of nitrated peptides from an equimolar mixture (ECP-1 and ECP-2 peptides), dissociation was performed using an acidic glycine buffer which transferred also the sample into the interface for ESI-MS analysis (Fig. 7.6). Biosensor association curves and subsequent dissociation of affinity-bound ECP peptides from a mixture of nitrated peptides are shown in Fig. 7.7a.

The ESI mass spectra of the affinity-eluate showed exclusively ions of the nitrated ECP-1 peptide (Fig. 7.7b). Affinity of the nitrated ECP peptide was found to be completely abolished upon replacing the positively charged residues Arg^{28}, Arg^{34}, Arg^{36}, and Lys^{38} by alanine, thus confirming the importance of cationic amino acids adjacent to the nitration site. Affinity determinations of the nitrated peptide in comparison to the intact ECP protein using the SAW biosensor provided also dissociation constants of approximately 6 nM for the nitrated peptide ECP (24-41) and 28 nM for intact ECP [37]. In addition to the characterization of sequence variations of ECP-nitrated peptides, a preliminary epitope motif derived from the antibody-binding affinities of the tyrosine-nitrated prostacyclin synthase peptides was shown. All these results reveal remarkable similarities, suggesting that (1) stabilization by adjacent positively charged residues and (2) surface exposition of tyrosine-nitrated sites are important factors [4].

The capability of anti-nitrotyrosine antibodies to discriminate between nitrotyrosine in different environments in proteins may be useful for producing antibodies to specific motifs containing tyrosine residues and for the development of highly specific biomarkers.

7.3.4 Proteolytic Affinity Excision-Mass Spectrometric Approach for Identification of Carbohydrate Recognition Structure in Human Galectins

Interactions of proteins with carbohydrates play an important role in several physiological processes such as cellular recognition processes, intracellular regulation pathways, immunological reactions, and the transcriptional or posttranscriptional regulation of gene expression. Galectins are a class of lectin proteins that typically bind β-galactose-containing glycoconjugates and share primary structural homology in their CRDs. Each galectin CRD recognizes different types of glycan ligands and shows highest affinity binding to different structures such as natural glycoconjugate ligands expressed on cell surfaces or in the extracellular matrix providing a large diversity of functions [3, 48]. Galectin-1 and galectin-3 have been shown to be involved in pre-mRNA splicing [51]; galectin-1 can induce apoptosis of activated T-cells by binding to cell surface oligosaccharides [32, 50], and galectin-3 can activate neutrophils [53]. The precise structure of galectins–glycoconjugate ligands interactions is not well understood. The combination of the proteolytic affinity excision approach and mass spectrometry was used to obtain the structural peptide sequence from CRDs of human galectin-1 and galectin-3 [19]. The newly developed approach was named CREDEX-MS (carbohydrate recognition domain excision mass spectrometry) and was performed using lactosylated Sepharose 4B as an immobilization matrix support. Human galectin-1 was bound first to the affinity matrix in phosphate-buffered saline, and any unbound protein was removed by washing steps. In situ proteolytic digestion of remaining bound galectin-1 was performed using trypsin, followed by complete removal of the unbound tryptic generated peptides. Elution was performed using buffer solution containing 0.3 M lactose.

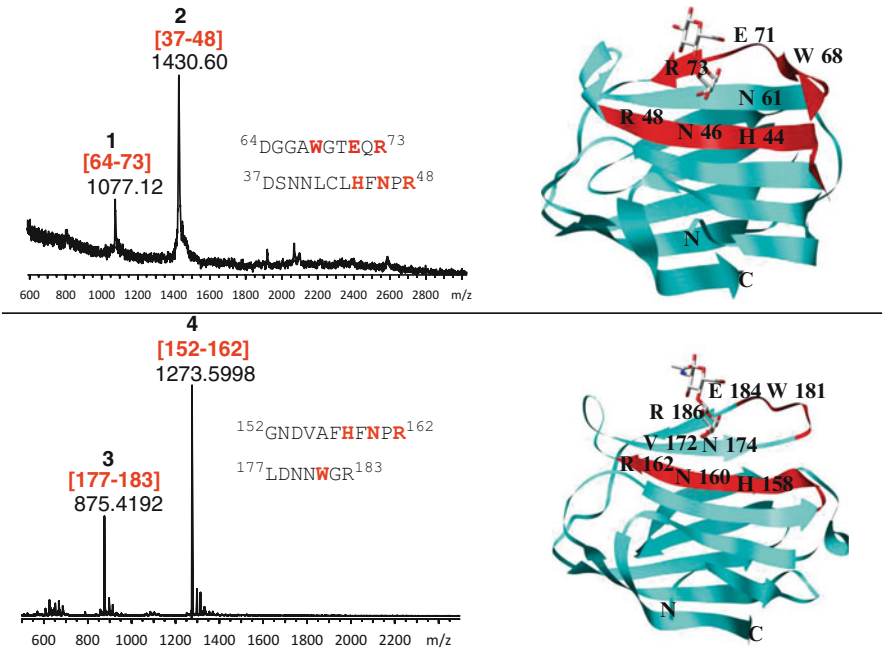

Fig. 7.8 Proteolytic CRDs' peptide excision for complexes of lactose with galectin-1 (*top*) and galectin-3 (*bottom*). MALDI-MS of elution fractions with signals of identified peptides. (*Right*) X-ray crystal structures (PDB entries 1W6O and 1A3K) showing the identified peptides in *red* and the amino acids that are in direct contact with the carbohydrate in *bold*. Reprinted with permission from Moise et al. J. Am. Chem. Soc. 2011; 133(38):14844-7. Copyright 2013 American Chemical Society

The MALDI-MS mass spectrum of the elution fraction shows two specific tryptic peptides (Fig. 7.8), which were displaced from bound galactin-1 by the presence of cognate sugar (lactose).

The two peptides (1 and 2) covered the galectin-1 (64-73) fragment, a sequence stretch with central tryptophan residue involved in C–H/π-interaction with galactose [19] and, respectively, galectin-1 (37-48) fragment [29]. CREDEX approach was applied for human galectin-3. Two specific peptides (3 and 4), galectin-3 (152-162) fragment, and galectin-3 (177-183) from the tryptic generated peptides were identified by MALDI-MS analysis of the lactose eluted fraction. The same two peptides were obtained by using proteolytic affinity extraction procedure, when galectin-3 was first digested in solution using trypsin; the resulted tryptic peptides were allowed to bind to the affinity matrix, and after washing step the bound peptides were eluted (Fig. 7.9).

Together these results provide evidence that the identified peptide fragments within CRDs of galectin-1 and galectin-3, respectively, comprise key amino acids in

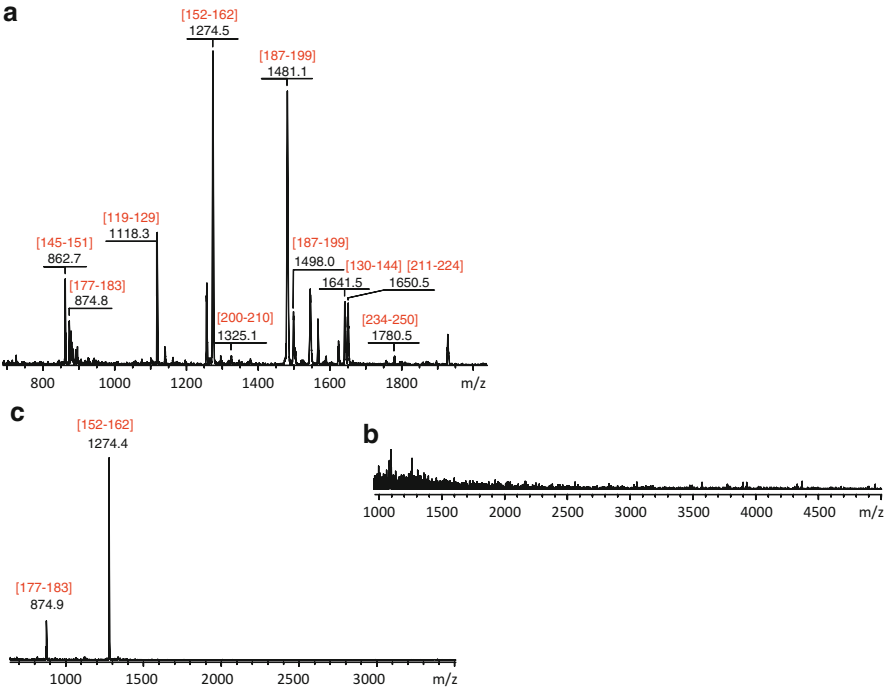

Fig. 7.9 Proteolytic CRDs' peptide extraction from galectin-3 digested peptide mixture. MALDI-MS of (**a**) supernatant fraction, (**b**) wash fraction, and (**c**) elution fraction identify the active galectin-3 peptide fragment (177-183) (peptide 3) and (152-162) galectin-3 peptide fragment (peptide 4). Reprinted with permission from Moise et al. J. Am. Chem. Soc. 2011; 133(38):14844-7. Copyright 2013 American Chemical Society

contact with the carbohydrate ligand. These peptide structures may be considered regions of biological importance for galectin-1 and galectin-3 and may be relevant for further studies of other types of sugar-binding proteins.

7.4 Summary

The results shown here provide evidence that the new affinity-mass spectrometric-based approach in combination with proteolytic digestion presents promising perspectives for elucidating (1) a high diversity of antigen–antibody interactions, (2) selective enrichment and identification of posttranslational modification in biological mixture, (3) extended binding sites, e.g., the interacting area for larger carbohydrates ligands. The new affinity-mass spectrometric approaches presented in this chapter may become increasingly utilized within interdisciplinary fields for

the development of (1) biomolecules that inhibit or propagate molecular cell processes, (2) structure-based drug design, and (3) rare diseases diagnostic approaches using defected enzyme–substrate affinities.

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