

A Novel Mass Spectrometric Epitope Mapping Approach without Immobilization of the Antibody

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Abstract

The development and application of a mass spectrometry-based epitope mapping procedure in solution and without immobilization of the antibody is described. Antigens were digested with proteases. Then size exclusion micro-column chromatography (SEC) was carried out prior to and upon exposure of the peptide mixtures to monoclonal antibodies. The epitope-containing peptide, affinity bound to the antibody and, thus, forming a stable complex eluted early as did all high-mass components, whereas all unbound low-mass peptides eluted late. Comparison of elution profiles in the presence and absence of the antibody showed a shift only for epitope-bearing peptides, enabling direct identification of the epitope. His-tag containing recombinant antigens Fibrillarlin and RA33 were used in combination with an anti-His-tag monoclonal antibody in order to develop the method. Application of this method for the determination of an epitope on RA33 against which a monoclonal antibody was directed identified the epitope sequence (⁸⁵IDGRVVEPKRA⁹⁵) using MS/MS peptide sequencing. Advantages of this approach include low sample consumption, few handling steps, and short duration of analysis. With our method we are ultimately aiming at developing a screening procedure to identify major epitopes in patients that may be suitable in the future for stratification of patients, needed for personalized therapies.

Keywords: Epitope mapping; Epitope extraction; Enzymatic proteolysis; Size exclusion chromatography; Matrix assisted laser desorption/ionization mass spectrometry; Monoclonal antibody

Abbreviations: AA: Amino Acid; DHB: 2,5-Dihydroxy Benzoic acid; RA33: Rheumatoid Arthritis auto-antigen33; ACN: Acetonitrile; TFA: Trifluoroacetic Acid; NH₄HCO₃: Ammonium bicarbonate; TBS: Tris Buffered Saline; BSA: Bovine Serum Albumin

Introduction

Epitope mapping is the process of identification of the molecular determinants for antibody-antigen recognition on the antigen. Identification of the epitope is a key step in the characterization of monoclonal antibodies, especially those used in therapeutic strategies (Nelson et al., 2000). It has also been essential in the design of vaccines against toxins (Kazemi and Finkelstein, 1991; Logan et al., 1991) or against enzymes (Gonzalez et al., 1994; Saint-Remy, 1997). To obtain information on sequential and/or assembled epitopes, various approaches have been developed in conjunction with mass spectrometry (Suckau et al., 1990; Hager-Braun and Tomer, 2005). Fine epitope mapping could be achieved by this approach with microgram quantities of antibodies and in a short time compared to conventional mapping methods (Dhungana et al., 2009; Macht et al., 1996; Parker et al., 1996; Suckau et al., 1990). The epitope analysis can be performed by epitope excision (Juszczak et al., 2009; Hager-Braun et al., 2006; Hochleitner et al., 2000; Suckau et al., 1990; Przybylski, 1994) or by epitope extraction (Tian et al., 2007; Bílková et al., 2005; Zhao et al., 1996; Zhao and Chait, 1994). In both cases the method is based on the fact that antibodies in general are very resistant to proteolytic digestion whereas the antigen has to be readily degradable.

In epitope excision studies, mass spectrometry was used to identify epitope peptides released from an immobilized antibody-antigen complex after limited proteolysis (Pimenova et al., 2009; Parker and Tomer, 2002; Suckau et al., 1990). Here, the antibody prevents either proteolysis (Jemmerson, 1996) or chemical modification (Burnens et al., 1987) of sites of the antigen that are situated in the antibody binding pocket. However, because of the steric hindrance between bound

antibody and proteolytic enzyme, the determined region is generally larger than the protected epitope that is recognized by the antibody as binding site (Manca, 1991). An important feature of this approach is that, because non-denaturing conditions are used, the antigen retains its native conformation so that assembled (conformational) epitopes can be determined (Parker and Tomer, 2000).

In epitope extraction, a protease-digested antigen peptide mixture is in most cases affinity-bound to an immobilized antibody. The epitope peptides are analyzed by MALDI-MS after washing off all unbound peptides and upon release of the epitope peptide from the immobilized antibody. Subsequently, sequential (linear) epitopes are identified directly by mass spectrometry (Bílková et al., 2005; Parker and Tomer, 2002).

In both approaches the peptides containing the epitopes are dissociated from the immobilized antibodies by elution with low pH buffer. The high accuracy and sensitivity of mass spectrometry helps in identifying the peptides that are regarded as epitopes. Furthermore, the resulting antibody-peptide complex must survive all procedures and washing steps prior to release of the epitope peptides. These mass spectrometry based methods in general require that the antibody be immobilized prior to reaction with antigen. Also critical to the success of this method is the choice of the proteolytic enzyme by which the antigen is being degraded and the already mentioned given stability of the antibody against proteolysis.

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In contrast to the procedure described above, an alternative epitope extraction procedure without immobilization of the antibody was described where epitope peptides were identified from a direct comparison of the MALDI mass spectra of the peptide control mixture with that following interaction with antibody. Disappearance of peptide ion signals in the mass spectra from the antibody reaction mixture was interpreted as a result of antibody-binding to the epitope sequences within the protein (Kiselar and Downard, 1999).

Our study presents a simplified approach that maps the epitope on a given antigen also without immobilization of the antibody and without a separate step to dissociate the epitope peptide from the antibody-peptide complex prior to mass spectrometric analysis. The antigen-antibody complex is maintained in solution throughout all steps needed for separating epitope-containing peptide from all non-binding peptides as affinity bound antibody complexes by size-exclusion chromatography. As size exclusion chromatography is a method readily applied to the analysis of intact molecular weights of antibodies and antibody conjugates (Brady et al., 2008; Lazar et al., 2005) the overall handling procedure is simple and straight forward. In contrast to previous reports, dissociation of the epitope peptides from the antibody occurs simultaneously to the preparation of the peptides for MALDI-MS analysis with acidic matrices in acidic solutions. Our novel epitope mapping approach was developed using a model system consisting of a commercially available monoclonal antibody directed towards the His-tag sequence present in recombinant Fibrillarlin and RA33 proteins. Our method was further applied successfully to a known system composed of a monoclonal antibody directed towards its sequential epitope on RA33 (El-Kased et al., 2009).

Materials and Methods

Proteins and antibodies

Recombinant Fibrillarlin protein derivative comprising amino acid range 76-321 of the full-length protein was a gift from the Institute of Immunology, University of Rostock, Germany. The protein was obtained in solution consisting of 50 mM sodium phosphate, pH 8, 0.3 M sodium chloride, 0.05 % Tween 20 and 8 M Urea, with a protein concentration of 1.21 mg mL⁻¹. Microcon® Centrifugal Filter Devices YM-10 [Millipore, USA] were used to desalt and concentrate the protein solution according to the manufacturer's protocol. 300 µl of protein solution were rebuffed and concentrated to a protein concentration of 2.25 mg mL⁻¹ in ammonium bicarbonate buffer, pH 8.5. The solution was then aliquoted (30 µl each) and stored at -20°C. Recombinant RA33 protein was purchased from Euroimmun AG, Luebeck, Germany. This autoantigen was shipped in solution consisting of 50 mM sodium phosphate, pH 7.4; 8 M urea; and 1000 mM sodium chloride, with a protein concentration of 2.42 mg mL⁻¹. 100 µl protein solution were quantitatively precipitated using 400 µl methanol/100 µl chloroform solution and resolubilised in 1000 µl 3 mM TRIS/HCl, pH 8.5, then aliquoted (30 µl each) and stored at -20°C. Penta-His antibody was purchased lyophilized from QIAGEN® GmbH [Hilden, Germany]. The lyophilized antibody (100µg) was dissolved in 100 µl distilled water resulting in a protein concentration of 1 mg mL⁻¹. Anti-histidine tag monoclonal antibody was purchased from AbD Serotec [Düsseldorf, Germany] in a phosphate buffered saline, pH 7.4, with a protein concentration of 1 mg mL⁻¹. Anti-RA33 monoclonal antibody (mouse anti-hnRNP-A2/B1) was purchased from Sigma [Munich, Germany]. The antibody was in solution consisting of 0.01 M phosphate buffered saline, pH 7.4 and 15 mM sodium azide, with a protein concentration of 1.5 mg mL⁻¹.

Proteolytic digests

Recombinant protein aliquots, dissolved in 30 µl of buffer (see above), were reduced with 3 µl 0.1 M freshly prepared dithiothreitol solution (15 mg DTT powder dissolved in 1 mL distilled water) at 56 °C for 30 min. Subsequently, freshly prepared iodoacetamide solution (6 µl; 18.5 mg IAA powder dissolved in 1 mL distilled water) was added at room temperature and incubated in the dark for 30 min in order to alkylate sulfhydryl groups. For in-solution tryptic digest, an enzyme solution of 1 mg mL⁻¹ in 3 mM TRIS/HCl, pH 8.5 was used [Promega, Madison, WI, USA]. 1.35 µl and 1.45 µl, respectively. Enzyme to substrate ratio was 1:50. In both experiments digestion was performed overnight at room temperature. For in-solution thermolytic digest; an enzyme solution of 1 mg mL⁻¹ in sodium acetate buffer, pH 8.5 was used [Sigma, Munich, Germany]. 1.45 µl enzyme solution were added to 30 µl RA33 solution with enzyme to substrate ratio of 1 : 50. The mixture was incubated overnight at room temperature. Protease inhibitors were not added as the expected stability of the antibody against proteases was suggesting to make this addition unnecessary except for one case which is discussed in the discussion section.

Incubation of proteolytic peptide mixtures derived from antigens with antibody prior to size exclusion

66.7 pmol anti-His antibody were incubated overnight at room temperature in two separate experiments with 17.5 pmol tryptic digest peptide mixture derived from Fibrillarlin and 17.5 pmol tryptic digest peptide mixture derived from RA33, respectively. 100 pmol anti-RA33 mAb were incubated overnight at room temperature with 35 pmol thermolytic digest peptide mixture derived from RA33.

Size exclusion chromatography of proteolytic peptides in the presence of the antibody

1 ml pipette tips (1000 µl blue tip, Greiner Bio-one GmbH, Frickenhausen, Germany) were used as microcolumns. First, a small filter paper [MoBiTec, Goettingen, Germany] was inserted manually to act as a frit. Second, 250 µl of the gel slurry [Superdex 30; GE Healthcare] were added after vigorous shaking. The compressed material height after gravity flow was approx. 8 mm (Figure 1). The supernatant was pushed through and discarded. Such prepared tips were first washed with 100 µl water and then equilibrated with 100 µl of the elution buffer. 50 mM ammonium bicarbonate, pH 8.5 [Fluka, Germany] and 10 % ACN / 0.1 % TFA, pH 2.2, respectively, were used as elution buffers. All solutions were loaded onto the top of the microcolumn using a pipette and the liquid was pressed through the microcolumn using an air-filled plastic syringe (volume: 5 ml). 10 µl of sample solvent was first added onto the compressed wet gel slurry and allowed to enter the gel bed. Next, 10 µl of elution buffer was added onto the column and pushed through to collect fraction 1 (void: a single drop (volume: 10 µl) is formed). During the entire procedure, the column is kept soaked all

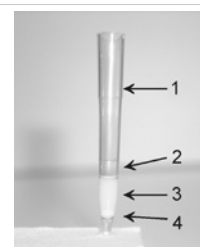


Figure 1: Assembly of the manual size exclusion column. 1: 1000 µl pipette tip, 2: supernatant after gravity flow, 3: compressed gel slurry (ca. 8 mm in height), 4: frit.

the time. Next, 10 μ l of elution buffer is added to collect fraction 2 as described above. This process is repeated successively for each further fraction.

SDS PAGE analysis

5 μ l of the sample solution (peptide mixture after digestion) were mixed with 5 μ l of 2-fold tricine SDS sample buffer. The 2-fold tricine SDS sample buffer contained 100 mM Tris-HCl, pH 6.8, 0.3 % SDS, 24 % glycerol (w/v), 8 % SDS (w/v), 5 % 2-mercaptoethanol (v/v) and 0.02 % Bromophenol Blue (w/v). This sample solution (10 μ l) was loaded onto a 16 % tricine gel, 1.00 mm x 10 well [Invitrogen, Karlsruhe, Germany]. The Broad Range Marker [New England BioLabs, Frankfurt/Main, Germany] as well as the Ultralow Range Marker [Sigma, Munich, Germany] were used for determining apparent molecular masses of the protein bands. Gels were run in tricine SDS running buffer (1 M Tris, 1 M tricine, 1 % SDS) (Schägger et al., 1988), using the Xcell Surelock™ MiniCell electrophoresis chamber [Invitrogen, Karlsruhe, Germany] at a constant voltage of 125 V for 60 min. Gels were fixed with a 50 % ethanol/10 % acetic acid/water solution and stained with colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988).

Western blot and Dot blot analysis

For Western blot analysis; 1D SDS PAGE was performed. Protein bands were blotted onto a PVDF membrane [Immobilon, Millipore, Schwalbach, Germany] by semi-dry blotting for 60 min with a current of 1.2 mA/cm². The membrane was cut into strips. After blocking the strips with 2 ml blocking buffer (TBS 5 % non-fat dry milk powder, 1 % BSA 0.02 % thimerosal) for 2 h, one RA33 protein strip was incubated with anti-RA33 mAb (1:1900) over night at 4 °C. A second RA33 protein strip and a Fibrillarin protein strip were incubated with the anti-His antibody (1:1900) overnight at 4 °C. Washing (three times, in TBS, 0.05 % TWEEN 20 (v/v), 0.1 % BSA (w/v), 0.02 % thimerosal (w/v)) was followed by secondary antibody reaction (biotin-GAH IgG + IgM anti-mouse, 1:20,000, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA/Dianova) for 1 h at room temperature. Another washing step and reaction with streptavidin peroxidase (1:10,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA/Dianova) was carried out for 1 h at room temperature. Detection was performed using the SuperSignal West Pico Chemiluminescent substrate [Pierce/ThermoFisher Scientific, Bonn, Germany]. Images were electronically processed to enhance band patterns.

For dot-blot analysis, fractions were collected after size exclusion chromatography of a mixture of anti-RA33 mAb that was incubated with thermolysin. The MINIFOLD blotting device [Schleicher & Schuell/Whatman, Dassel, Germany] was used and an antibody sample prior to elution was used as a positive control. After dot blotting, the membrane was taken out of the device and blocking as well as antibody incubation was performed as described above.

Mass spectrometry

For mass spectrometric peptide mapping, 0.5 μ l of the sample solution were spotted on a stainless steel target (Bruker Daltonik, Bremen, Germany) and 0.5 μ l of DHB matrix solution (12.5 mg/ml in 50 % ACN/0.1 % TFA) were added and mixed with the sample solution directly on the target. The mixture was then allowed air-dry. MALDI ToF MS measurements were performed using a Reflex III mass spectrometer [Bruker Daltonik, Bremen, Germany] equipped with the SCOUT source and operated in positive ion reflector mode. MALDI was carried out utilizing a nitrogen pulsed laser (337 nm, 3-5 ns pulse width). A total of 400 laser shots were accumulated producing sum spectra ranging from *m/z* 800-3500. Spectra were externally calibrated

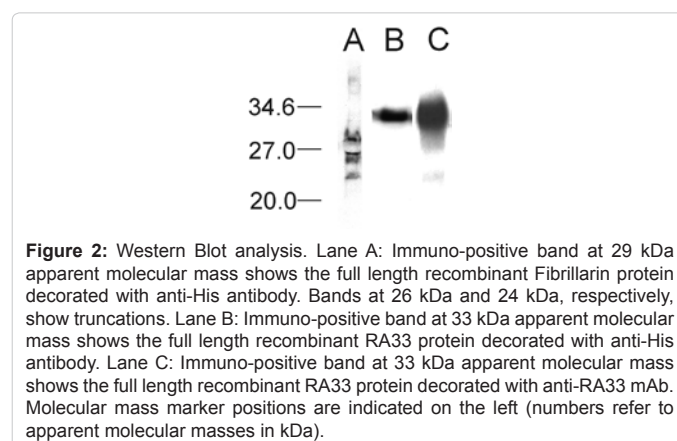
with a commercially available Peptide Calibration Standard [Bruker Daltonik, Bremen, Germany] then analyzed using FlexAnalysis 2.4 program [Bruker Daltonik, Bremen, Germany]. MS/MS spectra were acquired with an Axima MALDI QIT-ToF mass spectrometer [Shimadzu Biotech, Manchester, UK] in positive ion mode utilizing a nitrogen pulsed laser (337 nm, 3-5 ns pulse width) and employing a three-dimensional quadrupole ion trap supplied by helium (pulsed flow gas) for collisional cooling and argon (collisional gas) to impose collisionally induced dissociation (CID) (Koy et al., 2003; Koy et al., 2004). Spectra were externally calibrated with a manually mixed peptide standard consisting of bradykinin (1-7) [M+H]⁺ 757.39, angiotensin II [M+H]⁺ 1046.53, angiotensin I [M+H]⁺ 1296.68, bombesin [M+H]⁺ 1619.81, *N*-acetyl renin substrate [M+H]⁺ 1800.93, ACTH (1-17) [M+H]⁺ 2093.08, ACTH (18-39) [M+H]⁺ 2465.19, somatostatin [M+H]⁺ 3147.46, insulin (oxidized beta chain) [M+H]⁺ 3494.64. Further processing and analysis of the MS/MS spectra was performed with the Launchpad™ software, version 2.7.1 [Shimadzu Biotech, Manchester, UK].

Results

In order to develop and to apply our mass spectrometry-based epitope mapping procedure without immobilization of the antibody we investigated two recombinant proteins, a Fibrillarin derivative and RA33, for which monoclonal antibodies with different binding specificities were commercially available. As a prerequisite for the study we first checked antibody binding to the respective full-length antigen by Western blot. The recombinant proteins Fibrillarin and RA33 showed strong bands at apparent masses of 29 kDa and 33 kDa, respectively, showing the decoration of the full-length proteins with the respective antibodies (Figure 2). Fibrillarin, when stained by the anti-His-tag monoclonal antibody, showed also faint bands for truncated forms whereas RA33 staining was producing single bands. All band locations in the Western blot were in agreement with SDS-PAGE analyses of the full-length proteins (cf. Figure 3).

Next, in-solution proteolytic digests were performed in order to generate peptide mixtures, ideally with (all) peptides possessing molecular masses below 5,000 Da. These mixtures should contain the epitope sequences together with other non-epitope-containing peptides from the same protein or from other (contaminating) proteins in the sample, e.g. derived from impurities that may come from the expression systems. The success of the proteolysis reaction was monitored by both, SDS-PAGE (Figure 3) and mass spectrometric peptide mapping (Supplemental Figures 1, 2 and 3).

In-solution trypsin digest of recombinant Fibrillarin resulted



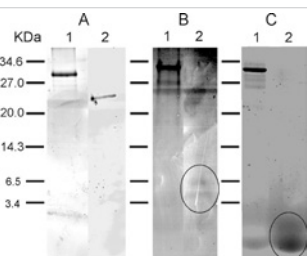


Figure 3: SDS-PAGE of recombinant proteins before and after enzymatic proteolysis. [A] 1D SDS-PAGE of recombinant Fibrillarlin protein. Lane 1: full length protein. Lane 2: band of trypsin-truncated Fibrillarlin. [B] 1D SDS-PAGE of recombinant RA33 protein. Lane 1: full length protein. Lane 2: band of trypsin proteolysis products of RA33 are circled. [C]: 1D SDS-PAGE of recombinant RA33 protein. Lane 1: full length protein. Lane 2: bands of thermolysin proteolysis products of RA33 are circled. Molecular mass marker positions are indicated on the left (numbers refer to apparent molecular masses in kDa).

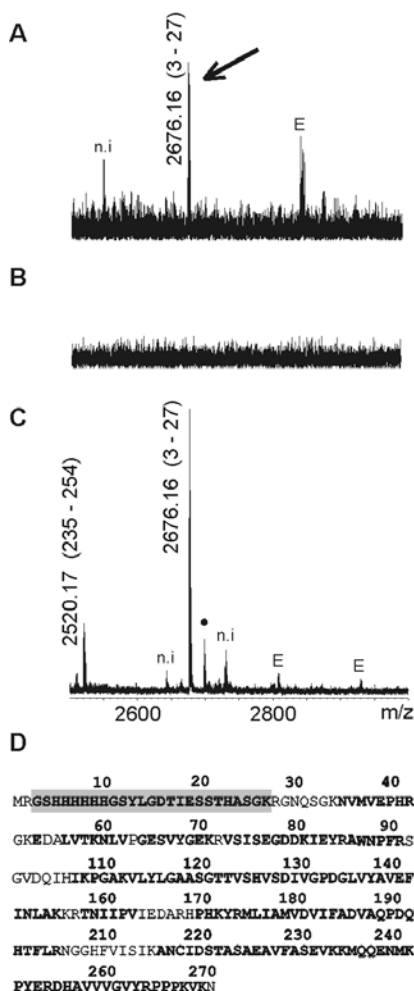


Figure 4: MALDI ToF MS peptide mapping analysis and amino acid sequence of Fibrillarlin. [A] Mass spectrum range m/z 2500–3000 shows Fibrillarlin epitope peptide in early eluting fraction (fraction 3) upon binding to anti-His antibody. [B] Mass spectrum of early eluting fraction (fraction 4) in the absence of anti-His antibody remains empty. [C] Mass spectrum of tryptic Fibrillarlin peptides. [D] Amino acid sequence in single letter code of recombinant Fibrillarlin showing sequence coverage after trypsin digest in bold letters. The shaded sequence displays the epitope motif that is recognized by the anti-His antibody. Matrix: DHB, n.i: not identified, E: derived from trypsin autoproteolysis, *: sodium adduct.

in partial proteolysis where the resulting peptide mixture showed incomplete digestion with a remaining protein band at approx. 24 kDa in the gel (Figure 3A) and with a singly charged molecular ion at m/z 23,603 in linear MALDI ToF MS [data not shown], indicating that Fibrillarlin is quite resistant to proteolysis. Nevertheless, in mass spectra ranging from m/z 775 to m/z 2520 (Table 1; Supplemental Figure 1) plenty of peptide ion signals were observed, resulting in nearly complete sequence coverage of recombinant Fibrillarlin (cf. Figure 4D). As mass spectrometric peptide mapping showed that peptides were produced that covered nearly the entire sequence of Fibrillarlin, we considered this mixture suitable for further investigations using size exclusion chromatography despite the fact that digestion was incomplete.

By contrast, tryptic digestion of RA33 resulted in complete proteolysis where the resulting peptide mixture showed ion signals in the mass range between m/z 800 and m/z 3000 (Table 2; Supplemental Figure 2) leading to nearly full sequence coverage (cf. Figure 5D). Accordingly, SDS PAGE showed faint and diffuse bands with maximal apparent masses of approx. 4 kDa (Figure 3B). Comparably, in-solution thermolysin digestion of RA33 resulted again in complete proteolysis of the starting material where the resulting peptide mixture showed ion signals in the mass range between m/z 600 and m/z 3000 (Table 3; Supplemental Figure 3), also leading to nearly full sequence coverage (Figure 6D). In agreement with mass spectrometry results, SDS PAGE analysis showed faint and diffuse bands with maximal apparent masses of ca. 3.5 kDa (Figure 3C). Accordingly, both RA33-derived digest mixtures were found well suitable for subsequent size exclusion experiments.

Size exclusion chromatography with a cut-off of roughly 4 to 5 kDa was applied in order to separate high-mass components, such as undigested starting material, protease, and, most importantly the antibody in the digest mixtures from the low-mass components, i.e. enzymatically produced peptides from the antigens. According to the size exclusion principle, high-mass compounds were expected to elute in early fractions (fractions 2 to 5 in our set-up; fraction 1 being the void) and low-mass compounds (below 4 to 5 kDa) should be present in late eluting fractions (fractions 6 to 10 in our set-up). Each experiment was performed such that for control and for investigating the elution profile of the proteolytic peptide mixture alone a first set of 10 fractions was collected with a volume of 10 μ l each. Peptides should only be found in the late eluting fractions. In the second set of elution fractions the peptide mixture was first incubated with the antibody and the same experimental conditions as mentioned above were applied for elution.

Sequence range	[M+H] ⁺ calcd	m/z (exp.)
3-27	2676.20	2676.16
35 - 42	981.49	981.49
35 - 50	1979.97	1979.98
43 - 50	1017.49	1017.49
53 - 59	775.42	775.42
60 - 71	1291.65	1291.63
73 - 85	1510.74	1510.74
86 - 91	790.40	790.43
94 - 112	1872.11	1872.13
113 - 145	3416.75	3416.67
149 - 155	786.47	786.47
158 - 168	1240.69	1240.67
175 - 194	2233.14	2233.10
195 - 205	1254.73	1254.73
206 - 215	1071.59	1071.57
216 - 234	1969.92	1969.91
216 - 235	2098.01	2098.01
235 - 254	2520.22	2520.17
236 - 254	2392.13	2392.10
255 - 268	1533.85	1533.87
265 - 270	1761.02	1761.91

Table 1: MALDI MS analysis of Fibrillarlin peptides after tryptic digest.

Sequence range	[M+H] ⁺ calcd	m/z (exp.)
15 - 20	836.43	836.70
15 - 21	964.52	964.78
21 - 37	1927.02	1927.45
22 - 37	1798.92	1799.33
38 - 45	1087.48	1087.72
46 - 53	993.49	993.73
46 - 58	1491.73	1492.07
62 - 88	2827.33	2827.95
99 - 111	1338.70	1338.96
112 - 119	861.56	861.81
112 - 128	2008.06	2008.50
113 - 128	1879.97	1880.39
120 - 128	1165.52	1165.78
129 - 136	1050.44	1050.68
129 - 146	2220.07	2220.10
137 - 146	1188.65	1188.90
152 - 167	1851.87	1852.27
152 - 172	2433.26	2433.77
153 - 167	1695.76	1696.13
153 - 172	2277.16	2277.66
173 - 184	1410.69	1411.02
173 - 185	1538.78	1539.13
186 - 199	1648.81	1648.20
190 - 199	1221.55	1221.84
200 - 212	1313.60	1313.87
203 - 212	1013.44	1013.70
213 - 227	1377.63	1377.94
228 - 237	912.38	912.63
277 - 284	781.36	781.61
285 - 309	2189.91	2189.37

Table 2: MALDI MS analysis of RA33 peptides after tryptic digest.

Hence, a comparable set of 10 fractions was obtained. The major difference between the two sets of data should be a shift in migration of the epitope peptide. This peptide was expected to be pulled through the column when bound to the antibody during filtration, now ending up in an early eluting fraction. All fractions from each set were analyzed by MALDI MS in the mass range of m/z 800 to m/z 3500.

Accordingly, for Fibrillarin epitope mapping, the elution behavior of the epitope containing peptide was different in the presence of the monoclonal anti-His antibody during filtration as compared to the elution profile in the absence of the antibody. After incubation with the anti-His antibody the epitope peptide appeared in an early eluting fraction (Figure 4A). In the absence of the antibody, as expected, no peptide ion signals were observed in mass spectra from early eluting fractions (Figure 4B), which stands in contrast to the mass spectra that were obtained from higher numbered (late eluting) fractions [data not shown]. The epitope peptide at m/z 2676.16 which was absent in the control experiment but was observed in the peptide mixture before SEC (Figure 4C) was further investigated by MS/MS peptide sequencing and determined the amino acid sequence of the His-tag peptide covering the range aa 3–27 (³GSHHHHHHGSYLGDIESSTHASGK²⁷) of recombinant Fibrillarin [data not shown] and confirmed tryptic cleavage after arginine residue 2 and lysine residue 27.

For RA33 epitope mapping using the anti-His-tag monoclonal antibody, the epitope peptide eluted early together with its methionine-oxidation products in the presence of the antibody (Figure 5A). The epitope peptide at m/z 2093.13 was found to be covering the sequence range aa 1-16 (¹MSHHHHHHHSMERERK¹⁶). Chemically assisted fragmentation-MALDI (CAF-MALDI) and subsequent MS/MS measurements were used to confirm the sequence assignment [data not shown]. This peptide was completely absent in the late eluting fractions after antibody incubation (Figure 5B). Notably, the peptide ion signal was also not detectable in the peptide mixture before SEC (Figure 5C). The absence of the ion signal in the latter two samples can be explained by suppression effects and by low ionization yields of the peptide. Only in the rather pure early eluting fraction larger ionization yields were

obtained mounting in sufficient intensity of this peptide ion signal to be observable by MS. Again, trypsin was found to cleave the protein after lysine and arginine residues according to its known sequence specificity.

Since the developed procedure worked well with two different proteins and reliably isolated the His-tag peptide as the antibody-bound epitope, we went for a first application example and selected a monoclonal antibody that was directed against an internal sequence of the antigen RA33. Again, RA33 was first digested, this time with thermolysin. Thermolysin is a “non-sequence specific” enzyme which preferentially cleaves at the N-terminal side of bulky and aromatic residues Ile, Leu, Val, Ala, Met, and Phe (Keil, 1992), but was readily applicable in our experiments as it yielded in complete proteolysis, which is advantageous when the goal is to determine a specific epitope to the smallest possible number of amino acid residues, as long as the epitope

Sequence range	[M+H] ⁺ calcd	m/z (exp.)
1 - 12	1551.63	1550.54
2 - 12	1420.59	1419.81
2 - 19	2367.04	2366.54
12 - 21	1380.70	1380.92
12 - 26	1867.98	1867.36
30 - 36	808.36	808.74
30 - 46	2146.01	2146.43
30 - 50	2621.18	2621.70
37 - 46	1372.67 *	1372.70 *
46 - 62	1890.96	1890.88
50 - 62	1458.79	1459.57
51 - 62	1359.73	1360.73
52 - 64	1464.75	1465.69
53 - 65	1480.78	1481.64
57 - 65	1041.56	1041.68
65 - 76	1303.59	1303.74
80 - 88	1008.53	1008.78
85 - 94	1168.68	1168.50
85 - 95	1239.72	1240.01
96 - 109	1437.74	1437.79
96 - 112	1793	1793.62
98 - 110	1366.71	1366.70
114 - 130	2044.97	2045.40
119 - 127	1137.63	1137.69
119 - 131	1718.78	1718.40
128 - 137	1319.63	1318.72
132 - 142	1309.65	1309.60
143 - 155	1449.79	1449.73
143 - 156	1596.86	1596.93
157 - 167	1287.58	1286.68
159 - 164	745.28	745.87
159 - 167	1087.47	1086.70
159 - 169	1299.62	1299.49
160 - 165	697.28	696.81
160 - 168	1053.48	1053.31
169 - 176	1001.58	1002.25
171 - 176	789.43	789.09
171 - 181	1282.63	1283.24
176 - 186	1208.65	1208.52
184 - 192	1118.61	1118.70
187 - 205	2106.01	2106.37
187 - 207	2310.10	2310.25
187 - 217	3214.48	3214.71
188 - 206	2139.99	2140.42
193 - 208	1712.81	1712.66
196 - 206	1152.55	1151.69
207 - 218	1127.49	1127.75
207 - 226	1840.80	1841.19
209 - 218	923.40	924.16
209 - 226	1636.71	1637.08
209 - 239	2890.26	2889.73
218 - 225	732.33	732.26
219 - 239	1985.88	1986.24
227 - 239	1272.57	1272.72
263 - 280	2002.87	2003.44
280 - 312	3100.35	3100.89
280 - 314	3342.47	3342.67
286 - 312	2482.06	2482.02
286 - 314	2724.18	2724.79

*oxidation of tryptophan residue

Table 3: MALDI MS analysis of RA33 peptides after thermolysin digest.

is not destroyed by cleavage. The resulting epitope peptide eluted early in the presence of the antibody (Figure 6A). In the control experiment, i.e. in the absence of the antibody during filtration, the same peptide eluted late together with other non-epitope peptides (Figure 6B). For comparison, the mass spectrum of the thermolytic peptide mixture before SEC is shown (Figure 6C). This epitope peptide at *m/z* 1240.01 was further investigated using MS/MS peptide sequencing (Figure 7) which confirmed that the peptide comprised the range aa 85–95 (⁸⁵IDGRVVEPKRA⁹⁵) of RA33. This result stands in agreement with the previously assigned epitope using immuno-analytical methods coupled with mass spectrometry (El-Kased et al., 2009).

The here described experiments were accompanied by control runs in which all conditions of the size exclusion chromatography steps were tested in order to determine potentially interfering ion signals that may appear in mass spectra after elution (i) due to bleeding of the gel slurry during filtration (Supplemental Table 1), (ii) due to background signals derived from enzyme autoproteolysis during incubation (Supplemental Tables 2 and 3), or (iii) derived from partial degradation of the antibodies used in the study (Supplemental Table 4). The generated lists of background signals from these mock experiments were found helpful for defining the epitope peptide ion signals in the early eluting fractions after subtraction of all potential background signals.

In the end, the most important criterion for defining the epitope peptide was the shift in elution of a peptide upon binding to the antibody so that it was identified in the early eluting fraction where otherwise only high mass compounds appeared, despite being small enough to elute in late eluting fractions when unbound.

Discussion

Fibrillarin is a 34 kDa autoantigen, present in all nucleated cell types that derives its name from its localization to both the fibrillar center (FC) and dense fibrillar component (DFC) of the nucleolus (Ochs et al., 1985). As a component of all small nucleolar ribonucleoprotein particles (snoRNPs), Fibrillarin seems to be involved in nearly all major post-transcriptional activities in ribosome synthesis, the first steps of rRNA processing, pre-rRNA modification, and ribosome assembly (Tollervey et al., 1993). Fibrillarin is a target for the spontaneously arising auto-antibodies in systemic sclerosis autoimmune disease (Ssc; scleroderma) (Takeuchi et al., 1995). Similarly, the RA33 auto-antigen, present in rheumatic autoimmune diseases, carries its name according to its migration behavior in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and has been found to be identical to the protein named hnRNP A2/B1 (RefSeq accessions NP_002128 and NP_112533; Uniprot Accession number P22626). hnRNP proteins are associated with pre-mRNAs in the nucleus in complex 4 which forms part of the spliceosome (Steiner and Smolen, 2002). They are involved in pre-mRNA processing and other aspects of mRNA metabolism and transport. It has been reported that the presence of anti-RA33 antibodies may precede disease symptoms by one year (Steiner et al., 1996). Therefore they can provide diagnostic help early in disease, particularly when Rheumatoid factor (RF) is negative in patient samples. In conjunction with anti-cyclic citrullinated peptide antibody (antiCCP antibody) level determination, measuring anti-RA33 autoantibody levels in RA patients and in other autoimmune patients has become important in particular for diagnosing RA in the absence of RF and for estimating severity of the disease (Feist et al., 2007; Steiner and Smolen, 2002). As both proteins play their roles in autoimmune diseases it is of interest to identify epitopes to which autoantibodies from individual patients are directed. Therefore, epitope mapping approaches that are suitable for studying patient sample-derived autoantibodies are of high interest.

The functional integrity of an antibody in all steps of our epitope mapping procedure is a prerequisite for successful application. This was checked by dot blot analysis with fractions from size exclusion chromatography. Binding of the antibody to the antigen was found in early eluting fractions (Supplemental Figure 6), showing that the antibody survived the procedure intact and active. The widely accepted knowledge that antibodies in general are resistant to proteolytic digestion, however, appeared not to be absolute. We recommend to check each commercially available antibody by incubation with the protease of choice, as in one example the anti-His-tag antibody was partially digested generating two dominant peptide ion signals at *m/z* 1488.73 and *m/z* 2383.41 upon incubation with trypsin (Figure 8). Both peptides were analyzed further using mass spectrometric peptide sequencing and confirmed that the generated peptides were part of the IgG heavy chain variable region and of the IgG light chain variable region, respectively [data not shown]. The proteolytically nicked antibody subsequently lost its capability to bind to its antigen and, hence, was not applicable for epitope mapping. It is of note to mention that the antibody-derived peptides (Figure 8) were observed

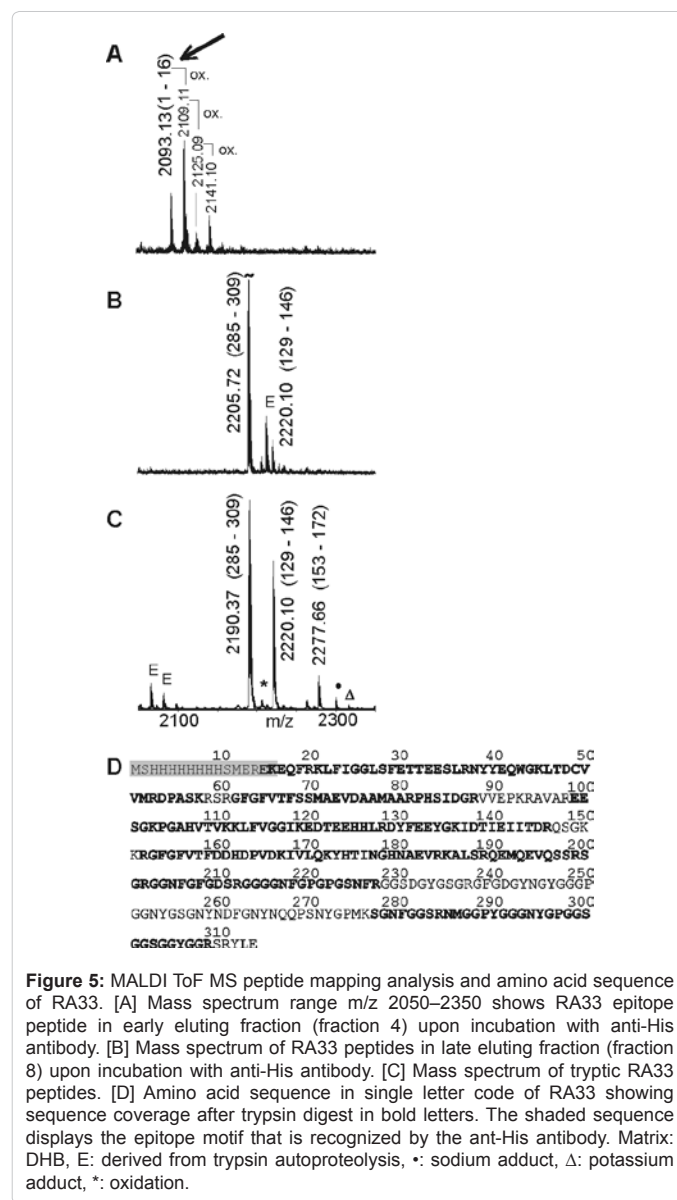
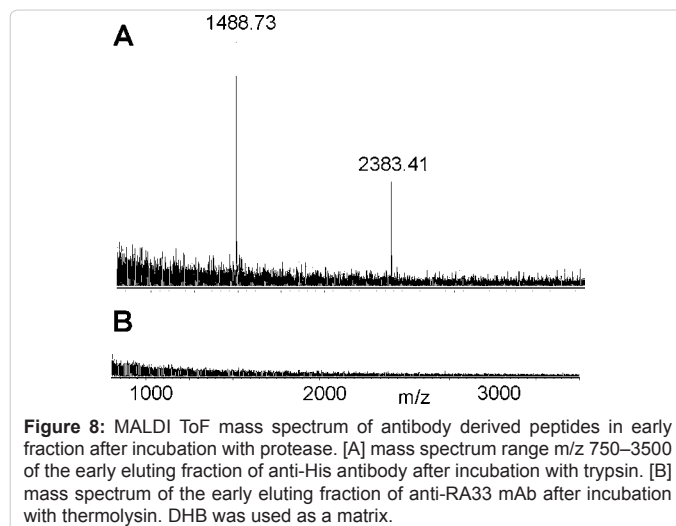
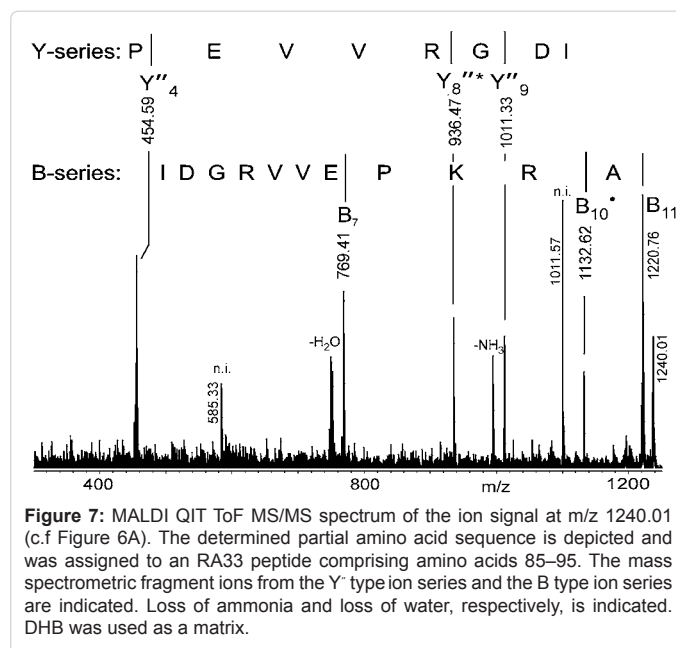
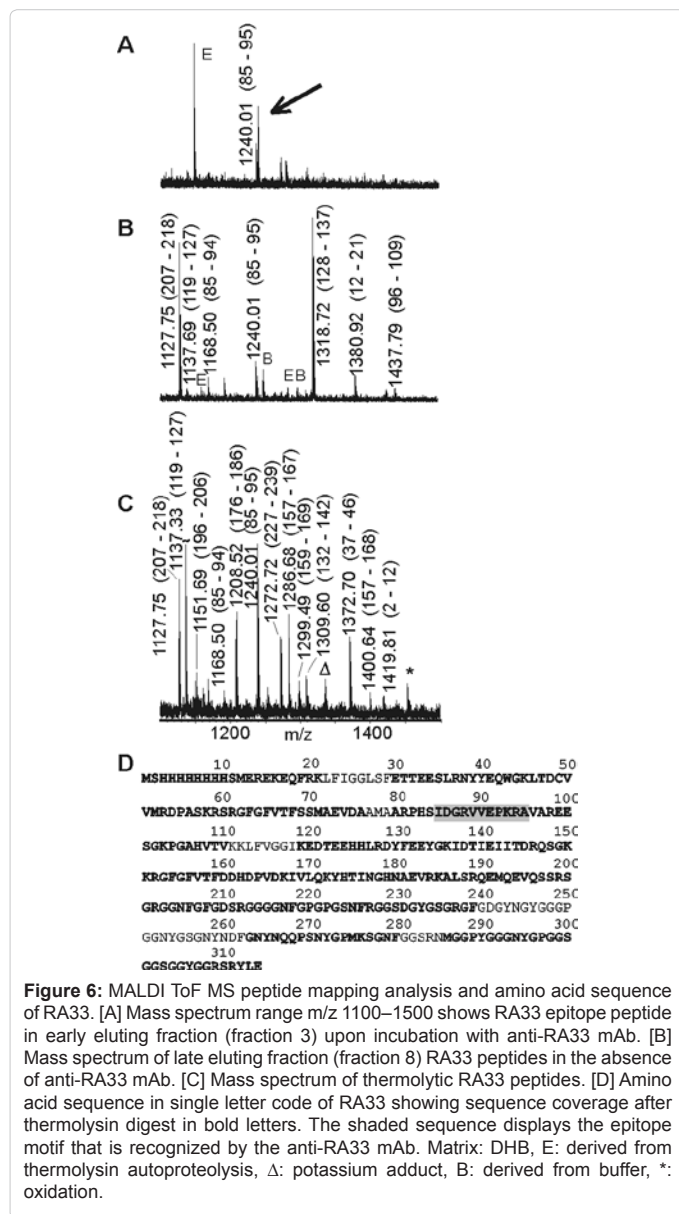


Figure 5: MALDI ToF MS peptide mapping analysis and amino acid sequence of RA33. [A] Mass spectrum range *m/z* 2050–2350 shows RA33 epitope peptide in early eluting fraction (fraction 4) upon incubation with anti-His antibody. [B] Mass spectrum of RA33 peptides in late eluting fraction (fraction 8) upon incubation with anti-His antibody. [C] Mass spectrum of tryptic RA33 peptides. [D] Amino acid sequence in single letter code of RA33 showing sequence coverage after trypsin digest in bold letters. The shaded sequence displays the epitope motif that is recognized by the anti-His antibody. Matrix: DHB, E: derived from trypsin autoproteolysis, *: sodium adduct, Δ: potassium adduct, *: oxidation.

in early eluting fractions. While this fact may seem a contradiction to our statements, we argue that this observation can be perfectly explained by the almost intact tertiary structure of the antibody, even after some peptide bonds were cleaved by the protease. As all steps were performed under non-denaturing conditions, the proteolytically nicked antibody eluted in early fractions as expected. Non-covalently attached peptides of the antibody were dissociated only upon addition of the acidic matrix through which the antibody structure was denatured. It should be noted, that the mentioned peptides were also observed in late eluting fractions, indicating that at least some parts of the antibody-related peptides already dissociated from the antibody within the timeframe of the experiment. By contrast, a different and more robust anti-His-tag antibody as well as the anti-RA33 mAb proved to be stable against proteolysis under the employed conditions and, therefore, both antibodies were found suitable for our peptide mapping procedure.

Preferably for our method, the proteolytic enzyme of choice should produce peptides of masses below 4 kDa to ensure a shift in elution upon binding to the antibody as compared to the free peptide. At the



same time, the presence of high-mass compounds do not interfere with the epitope mapping procedure as long as they do not contain all of the epitope sequences. This is due to the fact that the mass spectrometric measurements were tuned to the (low) mass range of interest below m/z 4,000. Because with peptides in that mass range MS/MS peptide fragmentation normally can be readily performed using the epitope peptides from the early eluting fractions, sequence specificity of the protease is not a major concern in epitope mapping using our method. The advantage of sequence specificity of proteases for easy assignment of peptide cleavage products by mass spectrometric peptide mapping may in fact turn out as a disadvantage for most epitope extraction cases, as especially the amino acids R and K (trypsin cleavage sites) are frequently found in epitopes. In fact, in our investigations the use of trypsin was found disadvantageous for mapping the epitope against which the anti-RA33 mAb is directed, as mass spectrometric peptide mapping showed that this protease destroyed the epitope. By contrast, using less sequence specific proteases ought to be preferable for epitope mapping with the procedure described here, as these proteases tend to produce more structure-specific cleavage products, particularly when

applied under so-called limited proteolysis conditions (Bantscheff et al. 1999).

Increasing the volume of the gel material to 400 μ l lead to an increase in the compressed height after gravity flow, leading to a longer path for the sample to move (data not shown). In this case, separation of peptides from antibodies was also successful but with a delay in elution so that at least 20 fractions had to be collected. Increasing the gel volume required increased pressure for elution which increased the risk of gel leaking, a major source of run-to-run elution variabilities.

In our hands, the gel volume of 250 μ l turned out to be well to handle without increased risk of leakage and with sufficient reproducibility.

Estimating the adequate sample amount is an important step for obtaining reliable results with this size exclusion chromatography-based method. Sample overloading leads to escape of peptides to early fractions. In our approach using a sample volume of ca. 4 % of the total bed volume and a peptide amount up to 35 pmol was found adequate to obtain optimal elution profiles. Our experimental procedure shows that incubation at a 3:1 ratio of antibody to peptides is well suitable for epitope mapping. This rather fixed antibody/peptide ratio differs from those reported in the literature where excess of antibody varies in broader ranges (Pimenova et al., 2009; Dhungana et al., 2009; Zhao and Chait, 1994) while in other studies peptides were used in excess (Bilková et al., 2005; Parker and Tomer, 2002; Peter and Tomer, 2001; Kiselar and Downard, 1999; Papac et al., 1994).

In addition to the results reported here, the epitope mapping procedure was investigated without reduction and alkylation of cysteine residues prior to enzymatic proteolysis. We obtained the same results (data not shown). This finding is explained by the fact that both, recombinant RA33 and recombinant Fibrillarín, contained only one and two cysteine residues, respectively, that were not involved in disulfide bond formation. Also, as was found in our studies, these cysteine residues were located far away from the epitope regions. In case of using other proteins with more cysteine residues, i.e. disulfide bonds, we recommend performing reduction and alkylation to facilitate complete proteolysis, so that rather small peptides are being generated, suitable for the size exclusion chromatography-dependent procedure. For epitope extraction experiments, as described here, this approach seems more generally applicable, and hence, we report the procedure including a reduction and alkylation step. By contrast, in epitope excision experiments, the fear of loss of folding and conformation might be more of concern and reduction and alkylation of cysteine residues ought not to be performed a priori.

Each experiment was performed in duplicate using two elution buffers, NH_4HCO_3 , pH 8.5 and 10 % ACN/0.1 % TFA, pH 2.2, respectively. There were marginal differences in the elution profiles and in general, the early eluting fractions were containing less peptides when elutions were performed with acidic buffers. This can be explained by slightly harsher conditions in the mild acidic medium that washed off unspecifically bound peptides while the specific interactions between epitope peptides and the paratopes still remained.

The peptide length for the epitope ($^{85}\text{IDGRVVEPKRA}^{95}$) as determined in this report is in fact shorter than the mentioned 15mer ($^{78}\text{MAARPHSIDGRVVEP}^{92}$) from our earlier paper (El-Kased et al., 2009). However, both sequences share the motif $^{86}\text{DGRVVEP}^{92}$ that was confirmed to be the epitope of the anti-RA33 mAb upon peptide sequence alignments (see Table 3 in El-Kased et al., 2009).

With our method, dissociation of the antigen-antibody complex

prior to mass spectrometric analysis is unnecessary because dissociation occurs during sample preparation of the collected fraction material onto the MALDI target simultaneously to the crystallization process of the MALDI matrix, leading to a decrease in sample handling and making it suitable for screening purposes in a clinical environment. We are ultimately aiming at a screening procedure to identify major epitopes in patients that may be suitable in the future for stratification of patients. Hence, reproducibility, ease of handling, minimal sample consumption as well as robustness of the method, as is the case with our method, are of utmost importance.

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