

# Epitope mapping on bovine prion protein using chemical cross-linking and mass spectrometry

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An analytical strategy for the analysis of antigen epitopes by chemical cross-linking and mass spectrometry is demonstrated. The information of antigen peptides involved in the binding to an antibody can be obtained by monitoring the antigen peptides modified by a partially hydrolyzed cross-linker in the absence and in the presence of an antibody. This approach was shown to be efficient for characterization of the epitope on bovine prion protein bPrP(25–241) specifically recognized by a monoclonal antibody, 3E7 (mAb3E7), with only a small amount of sample (200 picomoles) needed. After cross-linking of the specific immuno complex, a matrix-assisted laser desorption/ionization (MALDI) mass spectrometer equipped with an ion conversion dynode (ICD) high-mass detector was used to optimize the amount of cross-linked complex formed at 202 kDa before proteolytic digestion. To identify the cross-linked peptides after proteolysis without ambiguity, isotope-labeled cross-linkers, disuccinimidyl suberate (DSS-d0/d12) and disuccinimidyl glutarate (DSG-d0/d6), together with high-resolution Fourier transform ion-cyclotron resonance mass spectrometry (FTICR-MS) were used. As a result, a complete fading of the peak intensities corresponding to the peptides representing the epitope was observed when bPrP/mAb3E7 complexes were formed. Copyright © 2007 John Wiley & Sons, Ltd.

**KEYWORDS:** antigen/antibody complex; mass spectrometry; cross-linking; epitope mapping; high-mass MALDI-TOF MS; nano-LC-ESI-FTICR MS

## INTRODUCTION

Development of new analytical strategies for determination of protein/protein binding sites such as antigen epitopes is crucial to optimize immunoassay-based diagnostic tools. In this study we have evaluated a new analytical approach for characterizing epitopes using the monoclonal antibody 3E7 and bovine prion protein bPrP(25–241). Prions were first described in 1982 by Prusiner and defined as 'proteinaceous infectious particles'.<sup>1</sup> They are responsible for a class of neurological diseases termed *transmissible spongiform encephalopathies* (TSE).<sup>2</sup> To study the properties of prion proteins, a large number of monoclonal antibodies have been developed, allowing improvement of diagnostics and understanding of prion diseases. To characterize these monoclonal antibodies, an important step is determination of the epitopes, the precise region of the prion protein involved in the interaction within the immuno complex.

In this study we used the combination of chemical cross-linking and mass spectrometry (MS) to characterize

the epitope of the monoclonal antibody 3E7 on bovine prion protein. MS in combination with chemical cross-linking is an alternative tool to high-resolution methods, such as X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) for obtaining three-dimensional protein structural information.<sup>3</sup> As previously reported, cross-linking of proteins provides low-resolution structure information such as distance constraints within a protein or topological maps of the interacting regions within a protein complex.<sup>4,5</sup> MS is a very sensitive and rapid analytical tool<sup>6,7</sup> for mapping the cross-linked sites, requiring only picomole quantities of material. By using cross-linking reagents, covalent bonds are created between reactive amino acid side chains that are located in close proximity of each other (i.e. within the length of the cross-linker) of proteins or protein complexes. The commonly used reactive functional groups targeted by commercially available cross-linkers are amines, carboxylic acids, sulfhydryls from cysteines and nonselective photoreactive groups that become reactive by exposure to UV light.<sup>3,8–11</sup>

There are several ways how a cross-linker can covalently bind to amino acid residues of a protein leading to proteolytic products classified as mono-link<sup>20</sup> (Type 0),<sup>12</sup>

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loop-link<sup>20</sup> (Type 1)<sup>12</sup> and cross-link<sup>20</sup> (Type 2).<sup>12</sup> A mono-link modification describes a peptide in which only one end of a cross-linker is attached to an amino acid residue and the second end is hydrolyzed; in the case of a loop-link, both reactive groups of a cross-linker are attached to the same peptide, whereas in a cross-link the two ends of a cross-linker are attached to two different peptides. A number of studies have been successfully performed using MS combined with cross-linking. For example, low-resolution, three-dimensional protein structures<sup>13–21</sup> and protein–protein interactions<sup>20,22–26</sup> have been probed. So far, this approach has been applied to model proteins, such as ubiquitin,<sup>14,19</sup> myoglobin,<sup>20</sup> cytochrome *c* and lysozyme,<sup>15,20</sup> human and bovine serum albumins (BSAs),<sup>17</sup> bovine rhodopsin<sup>16</sup> and to the following protein complexes, calmodulin/melittin,<sup>23,25</sup> ribonuclease S,<sup>24</sup> Colicin E7 DNase/Im7,<sup>20</sup> cytochrome P450/2E1 and cytochrome b5.<sup>22</sup> Even though, theoretically, there is no mass limitation for this analytical strategy, MS and cross-linking has generally been applied only to study interactions in protein complexes with molecular masses of less than 100 kDa. There are a number of reasons why it is difficult to analyze high molecular weight protein complexes: (1) it is difficult to characterize intact cross-linked high molecular weight protein complexes by standard MS owing to limitation in high-mass detection; (2) given the expected great complexity of all possible products from enzymatic proteolysis of cross-linked proteins, it is a challenge to unambiguously identify the observed cross-linked peptide species; (3) the analysis of mass spectrometric data obtained from such protein cross-linking experiments requires efficient software tools.<sup>3</sup>

Here we present an analytical strategy for epitope mapping of an antigen/antibody complex, which allowed us to rapidly obtain information of the parts on the protein surface involved in the interaction. The epitope of the immuno complex [2bPrP·mAb3E7] with a total molecular weight of 195 kDa was analyzed. For mapping molecular interfaces in protein complexes it is usually necessary to know the amino acid sequences of both interaction partners. However, in most antigen/antibody complexes, the amino acid sequence of the antibody is not known. Therefore, it is virtually impossible to identify the cross-linked peptides derived from intermolecular cross-linking of an antigen/antibody complex. The strategy presented here therefore focuses on the comparison of mono-link modified peptides derived from cross-linking bPrP with and without the monoclonal antibody. The peptide mass map of amino acid residues on the surface of bPrP modified with a cross-linker is expected to be different in the presence and in the absence of an antibody bound to bPrP. Upon binding of the antibody, some residues of both interaction partners will be buried in the epitope and will no longer be accessible to chemical modification. The bPrP amino acid residues near the epitope will find themselves in close proximity to reactive residues on the surface of the antibody. In this case, cross-links can be formed between antigen and antibody. Consequently, the epitope of the target protein can be identified by monitoring changes in the intensity of

peptides carrying mono-link modification with and without addition of an antibody.

In the first step of our method, the bovine prion protein alone or in complex with the antibody was treated with the mixture of nonlabeled and heavily isotope-labeled amine reactive cross-linkers disuccinimidyl suberate (DSS-d0/d12) and disuccinimidyl glutarate (DSG-d0/d6). These cross-linking reagents generate patterns that are easily observed by MS, which is very helpful in distinguishing cross-linker-modified peptides from unmodified peptides after proteolytic digestion. To monitor the efficiency of cross-linking and to optimize the total amount of the specific [2bPrP·mAb3E7] complex formed in the sample, a matrix-assisted laser desorption/ionization/time of flight (MALDI-TOF) mass spectrometer equipped with a high-mass detection system was employed. High-mass MALDI MS is a method of choice for a rapid, specific and sensitive detection of protein/protein interactions stabilized by cross-linking.<sup>27</sup> The spectra are easy to interpret, because singly charged ions are predominantly formed during MALDI ionization process. The cross-linked immuno complex was digested by chymotrypsin and analyzed by liquid chromatography-electrospray ionization-Fourier transform ion cyclotron resonance (LC-ESI-FTICR) MS to achieve the high resolution and mass accuracy necessary to assign the cross-linked peptides without ambiguity.<sup>28,29</sup> The mixture of deuterium-labeled cross-linking reagents with their undeuterated counterparts allowed unambiguous detection of the doublets associated with peptides carrying a linker.<sup>30</sup> Using this strategy, the epitope of mAb3E7 on bPrP was determined. Only 200 picomoles of bPrP and 50 picomoles of mAb3E7 (total amount) were necessary to identify the epitope.

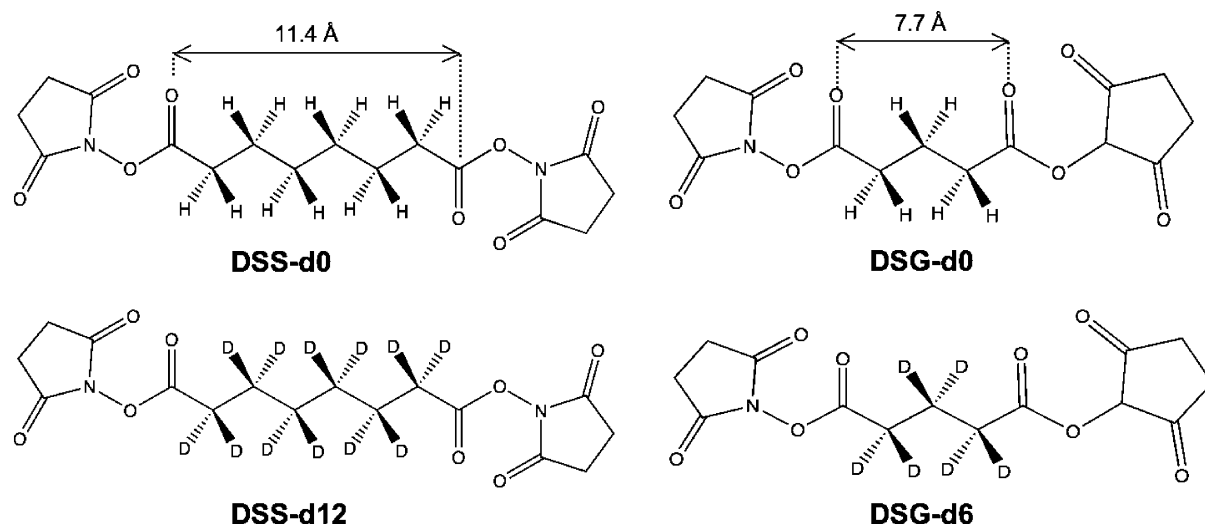
## EXPERIMENTAL

### Materials

Purified recombinant bPrP(25–241) was purchased from Prionics (Schlieren, Switzerland) in lyophilized form and dissolved in ultra pure water to reach a protein concentration of 1 mg/ml (43  $\mu$ M). This solution was aliquoted and stored in a freezer. A monoclonal anti-bPrP antibody, 3E7, was obtained from Roboscreen (Leipzig, Germany) in PBS buffer, pH 7.4, at a concentration of 1.9 mg/ml. The light cross-linking reagents DSS-d0 and DSG-d0 were purchased from Pierce (Rockford, IL, USA) and the heavy cross-linking reagents DSS-d12 and DSG-d6 were prepared as described<sup>20</sup> and stored in anhydrous dimethylformamide (DMF) at a concentration of 25 mM. Sequencing-grade chymotrypsin was purchased from Roche Applied Science (Penzberg, Germany). Sinapic acid for MALDI-TOF experiments and iodoacetamide were obtained from Fluka (Buchs, Switzerland). TCEP·HCl (tris(2-carboxyethyl)phosphine hydrochloride) was obtained from Pierce (Rockford, IL, USA).

### Instrumentation

High-mass MALDI-TOF mass spectra of the intact protein complexes were obtained using a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an HM1 high-mass detection system<sup>31</sup>



**Figure 1.** Chemical structures of the homobifunctional and isotope-labeled cross-linkers disuccinimidyl suberate DSS-d0 and DSS-d12 (spacer arm length 11.4 Å) and disuccinimidyl glutarate DSG-d0 and DSG-d6 (spacer arm length 7.7 Å).

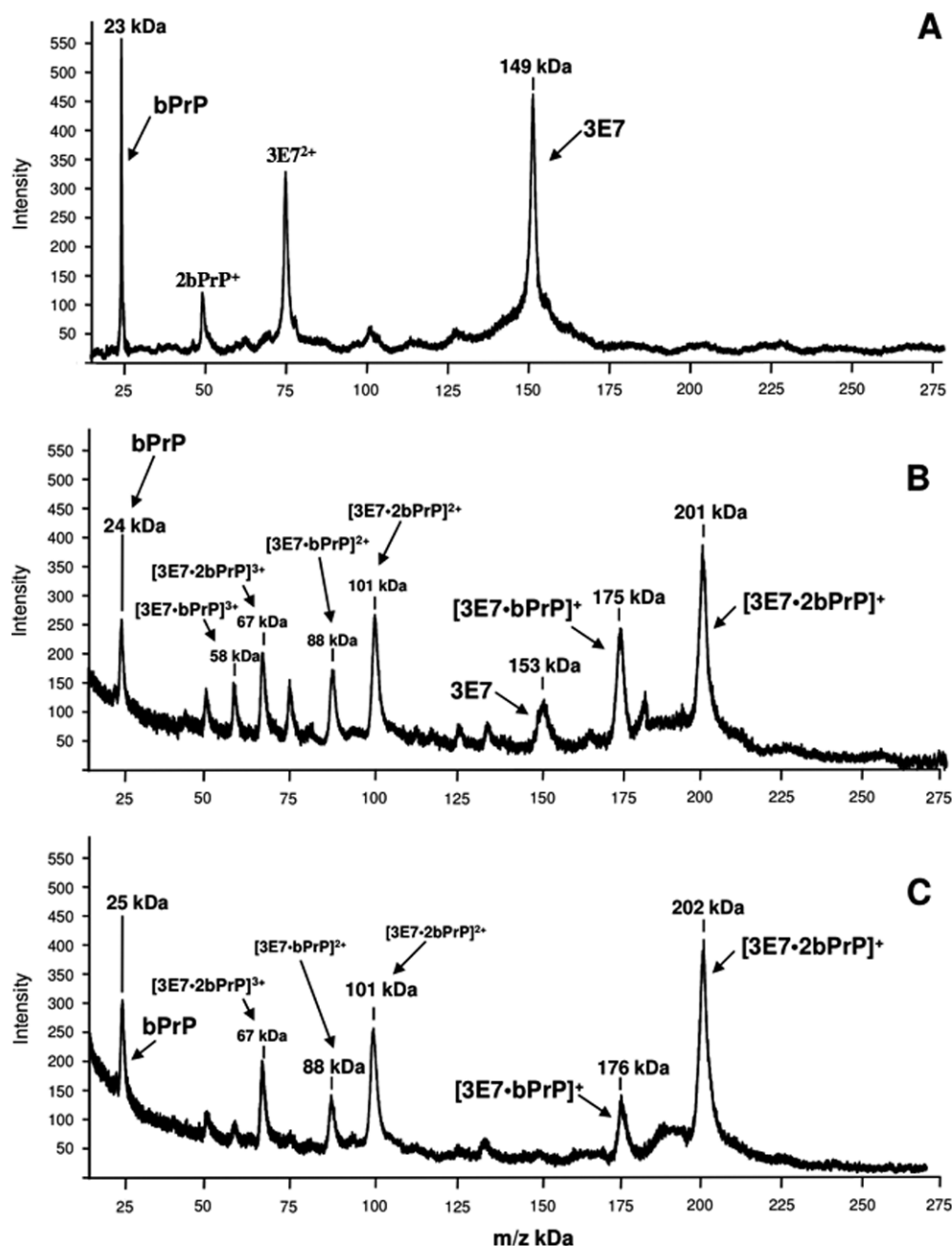
(CovalX, Zurich, Switzerland). Quantitative analysis of high-mass ions by MALDI is significantly better with the use of this high-mass detector than with standard detectors such as microchannel plates, because saturation by low-mass species is largely avoided. The instrument was operated in linear mode by applying an acceleration voltage of 25 kV. Mass spectra were acquired by averaging 200 shots. The laser pulse energy was adjusted slightly above threshold for ion production. The instrument was externally calibrated with a mixture of BSA and immunoglobulin G (IgG) each at a concentration of 10  $\mu\text{M}$ . The spectra for calibration exhibited spectra of BSA and IgG multimers with  $m/z$  66, 132, 150, 198, 264, 300 and 450 kDa. The mass accuracy was around 300 Da at 200 kDa.

Peptide mixtures after proteolytic digestions were analyzed by a commercial FTICR mass spectrometer (LTQ-FTMS, Thermo Finnigan, Bremen, Germany). This mass spectrometer is coupled on-line with a nano-high-performance liquid chromatography (HPLC) system and combines ion trap and FTICR technologies. In the first step, ions are accumulated by the linear ion trap and then transferred to the ion cyclotron resonance (ICR) cell, where the accurate mass measurements are carried out. Before analysis, the samples were cleaned up with ZipTip C18 pipette tips (Millipore, Billerica, MA, USA) according to the supplied user guide. Afterwards, a volume of 3  $\mu\text{M}$  of the samples was injected into an Eksigent-Nano-HPLC system (Eksigent Technologies, Dublin, CA, USA) by an autosampler and separated on a self-made reverse-phase column (75  $\mu\text{m} \times 80$  mm) packed with C18 material (AQ, 3  $\mu\text{m}$  200A, Bischoff GmbH, Leonberg, Germany). The column was equilibrated with solvent A (A: 3% acetonitrile; 0.2% formic acid in water). The mixture of peptides was injected into the column in solvent A with a flow rate of 0.5  $\mu\text{l}/\text{min}$  for 16 min. Then a washing step with solvent A was carried out for 5 min. Peptides were separated using the following gradient: 0–50 min, 0–60% B; 50–53 min, 60–97% B; 53–60 min, 97% B (B: 80% acetonitrile, 0.2% formic acid in water). The flow rate through the column

during the gradient was 0.2  $\mu\text{l}/\text{min}$ . Before the next injection the column was equilibrated with solvent A for 15 min. The mass spectral data was acquired in the mass-to-charge ( $m/z$ ) range from 300–2000. Data-dependent MS/MS spectra were recorded of the most intense ions (up to four, depending on signal intensity) using collision-induced dissociation (CID). Low-energy CID of the peptides was conducted in the ion trap. General mass spectrometric conditions for MS/MS acquisitions were as follows: normalized collision energy, 35%; ion selection threshold, 500 counts; activation  $q = 0.25$ ; and activation time 30 ms. Target ions already selected for MS/MS were dynamically excluded for 60 s.

### Cross-linking and sample preparation

Cross-linking experiments were carried out with isotopically labeled homobifunctional *N*-hydroxysuccinimide esters, DSS-d0/d12 and DSG-d0/d6. This class of reagents is commonly used to cross-link primary amines ( $\epsilon$ -amines on lysine residues and  $\alpha$ -amine groups on the *N*-termini of proteins) forming covalent amide bonds, but serine and tyrosine residues have also been described in previous publications<sup>32,33</sup> to be reactive towards *N*-hydroxysuccinimide esters. The difference between DSS-d0/d12 and DSG-d0/d6 is the number of deuterium atoms present and the reactive spacer length (Fig. 1). For cross-linking, 5  $\mu\text{l}$  of a 6  $\mu\text{M}$  solution of bPrP(25–241) and 5  $\mu\text{l}$  of a 3  $\mu\text{M}$  solution of mAb3E7 were mixed, and 1  $\mu\text{l}$  of a 3 mM solution of 1:1 mixtures of DSS-d0 and DSS-d12 or DSG-d0 and DSG-d6, respectively, in anhydrous DMF was added to achieve a 10, 20 or 50-fold molar excess of the cross-linking reagents over the total protein concentration. The optimal molar excess of the cross-linking reagents was 10-fold. The protein stock solutions (buffered only in the case of mAb3E7) were diluted with ultra pure water to reach the final concentrations. The pH of the reaction media was  $\sim 7$ . For the control sample, a solution of bPrP/mAb3E7, prepared as above but without the addition of cross-linking reagents, was used. The samples were incubated at room temperature, and after 1 and 2 h of incubation an aliquot



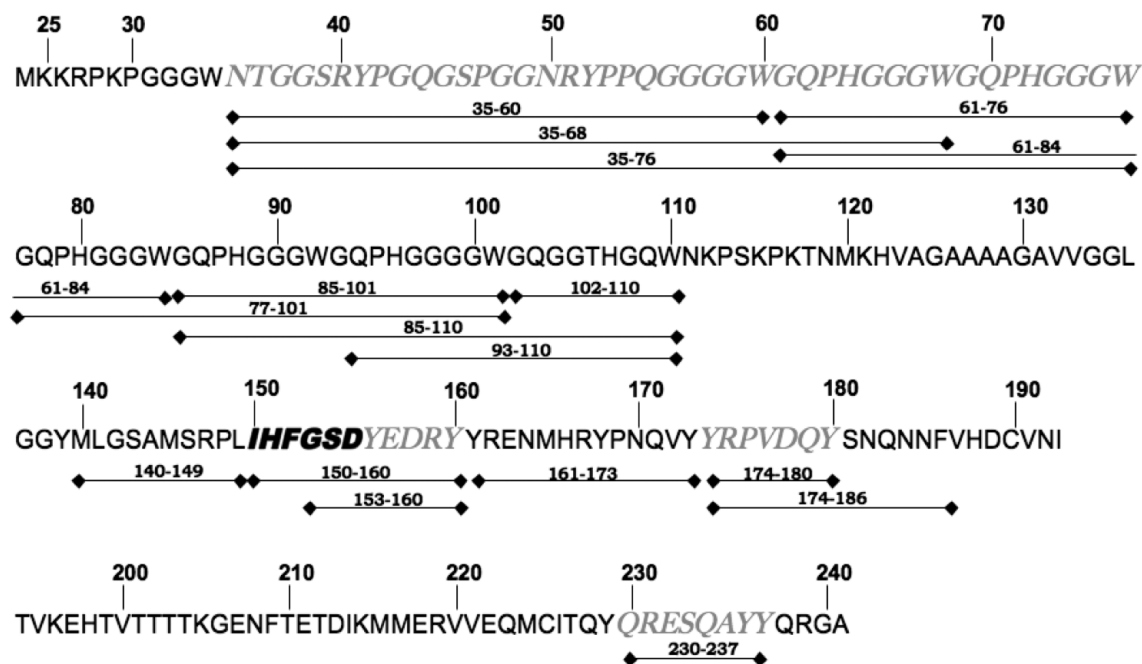
**Figure 2.** High-mass MALDI-TOF mass spectra of the immuno complexes formed between bPrP and the monoclonal antibody 3E7. (A) Mixture of bPrP and mAb3E7 analyzed without a cross-linking reagent. (B, C) Mixture of bPrP and mAb3E7 analyzed after the cross-linking reaction with DSG-d0/d6 after 1 h of incubation (B) and after 2 h (C). Molar ratio of bPrP : mAb3E7 was 2 : 1 and molar ratio of total bPrP+mAb3E7 : cross-linking reagent was 1 : 10 (see 'Experimental').

of the reaction mixture was taken for analysis. Formation of the bPrP/mAb3E7 complex was monitored by high-mass MALDI-TOF MS. Cross-linking reactions were quenched by adding an ammonium bicarbonate solution to a final concentration of 4 mM.

For MALDI-TOF MS analysis, the dried-droplet sample preparation technique was used: 1  $\mu$ l of the protein solution was mixed with 1  $\mu$ l of sinapic acid in 50% acetonitrile/0.1% trifluoroacetic acid (TFA) at a concentration of 10 mg/ml in an Eppendorf tube and 1  $\mu$ l of the mixture was spotted onto the MALDI plate.

### Chymotrypsin digestion

Before enzymatic digestion the proteins were reduced with TCEP·HCl (final concentration 2 mM), alkylated with iodoacetamide (final concentration 2 mM) and denatured in 10% acetonitrile and 2 M urea. The samples were then diluted with the digestion buffer (50 mM ammonium bicarbonate, pH 8.0). Sequencing-grade chymotrypsin was added to give an enzyme/protein ratio of 1/100 (w/w). Proteolysis was carried out at 25 °C overnight. The enzymatic reaction was stopped by lowering the pH to 2–3 by adding TFA to a final concentration of about 0.2%. These samples were then analyzed by nano-LC-ESI-FTICR MS.



**Figure 3.** Peptide mass fingerprint of bPrP(25–241) obtained after chymotryptic proteolysis. The analysis was performed using nano-LC-ESI-FTICR mass spectrometry and peptide identification was achieved using the Mascot search engine. The 17 peptides identified here represent a 60% sequence coverage. The epitope of mAb3E7 on bPrP(25–241) is [150–155] and marked in bold italics. The part of the bPrP(25–241) sequence presented in grey italics covers identified cross-linked peptides after digestion of bPrP modified either with DSS-d0/d12 or DSG-d0/d6 (31% of sequence).

**Table 1.** Molecular weights and calculated mass shifts for mono-links, loop- and cross-links for DSS-d0/d12 or DSG-d0/d6 modified peptides

Cross-linker	MW	Mass shift for mono-link	Mass shift for loop/cross-link
DSS-d0	368.35	156.08	138.07
DSS-d12	380.42	168.15	150.14
DSG-d0	326.26	114.03	96.02
DSG-d6	332.30	120.07	102.06

## RESULTS AND DISCUSSION

### Analysis of the intact protein complexes bPrP/mAb3E7

In order to generate a sufficient amount of cross-linked peptides after proteolysis of the immuno complex of interest for epitope mapping, it was crucial to optimize the yield of cross-linked specific complex. The proper molar ratios between all the components in the reacting mixture were optimized to be 2:1 for bPrP:mAb3E7 and 1:10 for total bPrP+mAb3E7:cross-linking reagent. The presence of the specific cross-linked immuno complex was confirmed by high-mass MALDI-TOF MS, which allows a sensitive and quick analysis of macromolecules in the 10–1500 kDa range. A bPrP/mAb3E7 mixture was cross-linked using the isotope-labeled cross-linkers DSS-d0/d12 and DSG-d0/d6 (Fig. 1). After incubation for 1 h, signals for the specific complexes corresponding to [mAb3E7-bPrP] and [mAb3E7-2bPrP] at 175 kDa and 201 kDa, respectively, were observed, and the mass peak representing the unbound antibody at 153 kDa

was still present (Fig. 2(B)). After 2 h, the peak corresponding to the doubly bound antigen [mAb3E7-2bPrP] became the major peak (Fig. 2(C)) and the peak which represents the unbound antibody was almost absent. This analysis indicated that the sample contains mainly the cross-linked specific complexes: [mAb3E7-2bPrP] and [mAb3E7-bPrP]. No nonspecific multimeric complexes were detected in the 10–1500 kDa range after the cross-linking reaction. The issue of specificity of the complexes formed has been discussed in detail in an earlier publication from our group by Nazabal *et al.*<sup>27</sup> Hence, successful cross-linking of bPrP and mAb3E7 can be ensured.

When comparing the mass spectrum of a control sample without cross-linking reagent (Fig. 2(A)) with the mass spectra of the cross-linked mixture of bPrP and mAb3E7 (Fig. 2(B), (C)), the signals corresponding to free bPrP and mAb3E7 strongly decrease after cross-linking, while new signals for cross-linked protein complex species arise. Introduction of mono-links and loop-links creates a mass difference equal to or greater than 1 kDa after 1 h of cross-linking, corresponding to about 3–4% of a protein's or a protein complex's molecular weight. The mass shifts due to modification of a protein with a single DSS-d0/d12 or DSG-d0/d6 unit (Table 1) fall into the ~100–170 Da range. Because of the limited resolution and mass accuracy in high-mass MALDI-TOF-MS, it is not possible to deduce the exact nature and number of cross-linker modifications. Only an approximate number of cross-linkers covalently attached to bPrP, to mAb3E7 and to bPrP/mAb3E7 complexes could be estimated. For instance, in the case of bPrP cross-linked with DSS-d0/d12, the mass difference between the control sample

and the cross-linked protein complex after 2 h of cross-linking was 2 kDa, corresponding to covalent modifications by about 11–14 molecules of DSS (mono-link, loop-/cross-link).

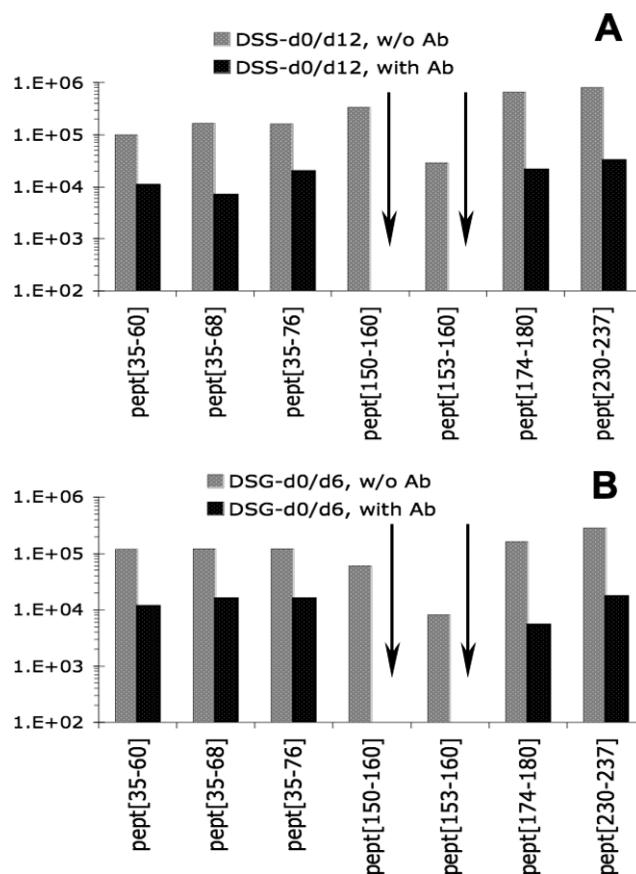
### Enzymatic digestion

bPrP and bPrP/mAb3E7 complexes were proteolytically digested to produce a set of cross-linked and noncross-linked peptides. These peptides were analyzed to determine the epitope. First, an enzymatic cleavage was carried out of unmodified bPrP and the conditions were optimized to obtain the highest possible protein sequence coverage. These conditions were applied to all later experiments with the cross-linked bPrP and the bPrP/mAb3E7 complexes. Usually, trypsin is the common enzyme for proteolytic digestion in protein chemistry because of its high specificity. However, the target amino acid residues, arginine and lysine, are unevenly distributed in the bPrP sequence. When trypsin was tested for digestion of bPrP, the sequence coverage was found to be below 30%. Therefore, another proteolytic enzyme was chosen, chymotrypsin. The theoretical peptides resulting from chymotryptic digestion were in the appropriate mass range (mostly between 400–2750 Da) for nano-LC-ESI-FTICR MS analysis. Despite the relatively low specificity of chymotrypsin, the protein sequence coverage was much higher (60%) compared to trypsin cleavage. PEP-SCAN analysis<sup>34</sup> of the mAb3E7 epitope suggests that the amino acids 150–155 of bPrP are involved in the binding to this antibody. Chymotrypsin is a pancreatic serine protease and it preferentially cleaves at the C-termini of tryptophane, tyrosine and phenylalanine, and in some cases at leucine, methionine and histidine.<sup>35</sup> Because of the epitope region of bPrP between amino acids 150–155, it is expected to be cleaved into two peptides by chymotrypsin, and a precise epitope analysis should be possible in this case.

The mass spectrometric data from a nano-LC-ESI-FTICR MS run was used for protein identification. Bovine prion protein was identified among other prion proteins of different origins with a high confidence (a Mascot score of 950 on average) and a sequence coverage of 60% (Fig. 3). All reported peptides covering the bPrP sequence were identified with Mascot scores greater than the identity threshold. Peptides with Mascot scores below the identity threshold were excluded from the analysis. The Mascot identity threshold statistically discriminates true positive from false positive peptide identifications. For determination of the mAb3E7 epitope, it is important to take into account the sequence coverage of mono-links identified after chymotryptic digestion of bPrP in cross-linking experiments. The higher the coverage, the better the chances to determine such peptides that are part of the epitope. From 17 identified bPrP peptides covering 60% of the protein sequence, 7 peptides were found to be modified as a mono-link, covering 31% of the sequence (Fig. 3).

### Analysis of the cross-linking products after enzymatic digestion

Cross-linking reactions of bPrP and bPrP in complex with the mAb3E7 before proteolysis were conducted with isotope-labeled cross-linkers DSS-d0/d12 and DSG-d0/d6 (Fig. 1), as



**Figure 4.** Comparison of the peak intensities of the modified bPrP peptides with DSS-d0/d12 (A) and with DSG-d0/d6 (B) on a logarithmic scale. The grey bars represent the average peak intensities for DSS-d0 and DSS-d12 (A) and for DSG-d0 and DSG-d6 (B) in the experiments without the antibody (Ab), while the black bars represent the peak intensities in experiments with the antibody. Peptides for which the intensity of the corresponding peaks in the presence of the antibody is fading are marked with arrows. Peak intensities below 500 au were considered as noise.

described in the experimental part. The expected  $m/z$  values corresponding to mono-links were calculated, and the entire LC-MS run was manually analyzed using the 'extracted ion chromatogram' option of XCalibur, the instrument-specific software. A slight shift to shorter chromatographic retention time was observed for peptides modified with a deuterium-labeled cross-linker. The analysis of all the identified bPrP peptides was focused on whether the expected mass shift due to cross-linker modification of amino acid residues such as Lys, Tyr and Ser was observed or not. A summary of unmodified bPrP peptides and of peptides that were found to be modified as mono-links is presented in Table 2 and in Fig. 4. Identification of peptides bearing a mono-link was based on the peptide precursor mass with a mass tolerance of  $\pm 2$  ppm, and on the specific isotopic signature due to the light and heavy cross-linker modification (6 mu for DSG-d0/d6, and 12 mu for DSS-d0/d12). In some cases (Table 2) the identified mono-links were additionally confirmed by searching the MS/MS data with the Mascot search engine using the SwissProt database, allowing modifications with

**Table 2.** Identification of bPrP peptides unmodified and modified with DSS-d0, DSS-d12, DSG-d0 and DSG-d6 as a mono-link

Unmodified peptides			Modified peptides			
A-A start-end <sup>a</sup>	<i>m/z</i> , (M + <i>nH</i> ) <sup><i>n+</i></sup>	Tyr/Ser <sup>b</sup>	Identified mono-link, DSS-d0	Identified mono-link, DSS-d12	Identified mono-link, DSG-d0	Identified mono-link, DSG-d6
35–60	854.39 <sup>3+</sup>	2Y/2S	<b>906.42<sup>3+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>	<b>910.44<sup>3+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>	<b>892.40<sup>3+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>	<b>894.41<sup>3+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>
35–68	835.13 <sup>4+</sup>	2Y/2S	#874.15 <sup>4+</sup>	<b>877.17<sup>4+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>	<b>863.64<sup>4+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>	<b>865.15<sup>4+</sup> (Y7)</b> <b>(S12)</b> <b>(Y18)</b>
35–76	823.57 <sup>5+</sup> 1029.22 <sup>4+</sup>	2Y/2S	854.79 <sup>5+</sup>	857.20 <sup>5+</sup>	846.38 <sup>5+</sup>	847.58 <sup>5+</sup>
140–149	531.78 <sup>2+</sup>	2S	–	–	–	–
150–160 <sup>c</sup>	467.87 <sup>3+</sup> 701.31 <sup>2+</sup>	2Y/1S	<b>519.90<sup>3+</sup> (Y7)</b>	#523.92 <sup>3+</sup>	<b>505.88<sup>3+</sup> (Y7)</b>	<b>507.89<sup>3+</sup> (Y7)</b>
153–160 <sup>c</sup>	502.70 <sup>2+</sup>	2Y/1S	580.74 <sup>2+</sup>	586.78 <sup>2+</sup>	559.72 <sup>2+</sup>	562.73 <sup>2+</sup>
161–173	590.61 <sup>3+</sup>	3Y	–	–	–	–
174–180	470.73 <sup>2+</sup>	2Y	548.77 <sup>2+</sup>	554.81 <sup>2+</sup>	527.75 <sup>2+</sup>	530.77 <sup>2+</sup>
230–237	522.74 <sup>2+</sup>	1Y/1S	600.78 <sup>2+</sup>	606.82 <sup>2+</sup>	579.76 <sup>2+</sup>	582.78 <sup>2+</sup>

<sup>a</sup> Number of amino acid residues in the identified peptides from chymotryptic digested bPrP.

<sup>b</sup> Presence and number of tyrosines (Tyr, Y) and serines (Ser, S) residues in the particular peptide.

<sup>c</sup> Peptides that are part of the bPrP epitope.

– these peptides were not modified with the cross-linker.

All reported unmodified peptides were identified by MS/MS data with Mascot scores greater than the identity threshold, and all the modified peptides are in bold. The monoisotopic precursor *m/z* ions selected for CID are shown with their charge states. The peptides marked with # were below the identity threshold, but their isotopic partner was identified on both MS<sup>1</sup> and MS<sup>2</sup> levels. The mono-linked peptides identified only on the MS<sup>1</sup> level due to the characteristic isotopic shifts are in italics. The exact modified amino acid in the peptides [35–60] and [35–68] could not be assigned.

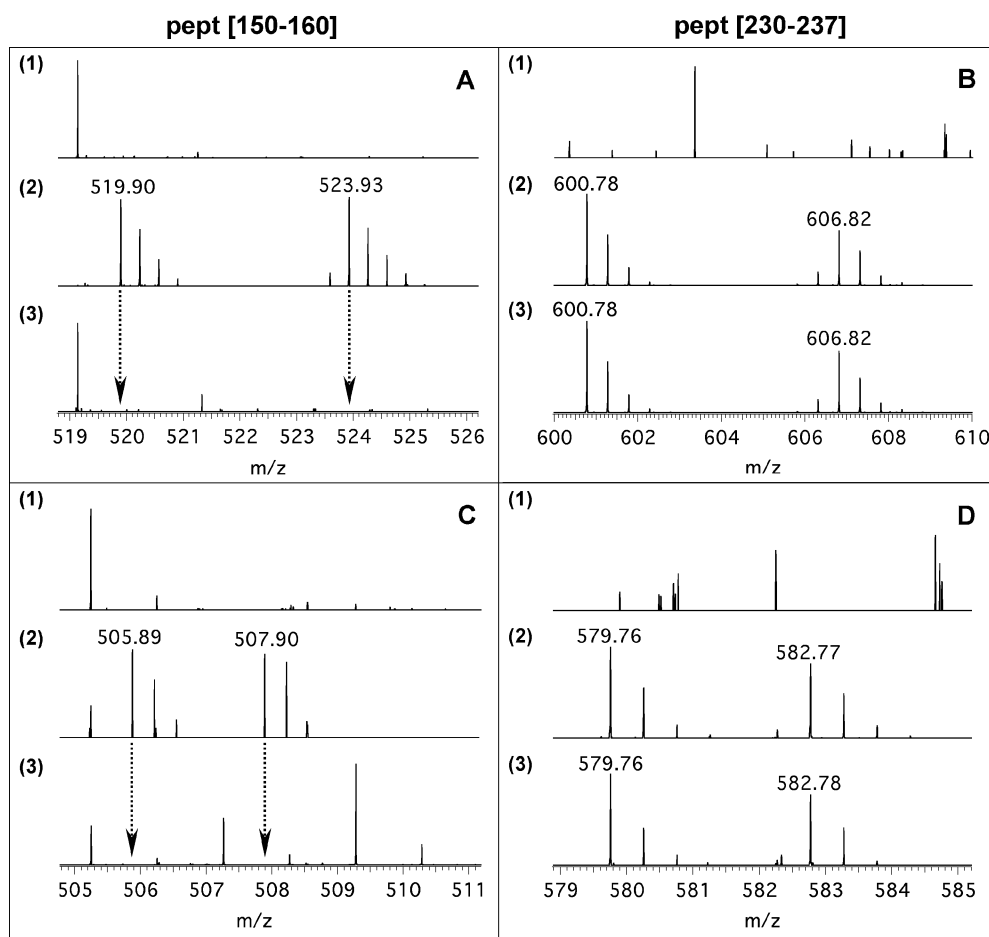
DSS-d0/d12 and DSG-d0/d6 at Lys, Tyr and Ser residues. The amino acid residues that are shown in parentheses in Table 2 most likely carry the mono-link modification, based on Mascot assignment. MS/MS analysis could often pinpoint the exact location of chemical modification; in cases where several amino acid residues are reactive towards the cross-linker, a roughly equal probability of modification was found by Mascot.

### Determination of the peptides representing the epitope

The antibody sequence is unknown, which prevents us from determining a cross-link product between bPrP and mAb3E7 using proteomic data mining. Detection of a cross-link representing the protein–protein contact site in the complex mixture of the digested immuno complex is difficult owing to its unknown mass. MS/MS assignment of such peptides is possible only if both protein sequences are known. The approach we describe here allows epitope analysis by studying mono-linked peptides of the interaction partner with a known sequence, bPrP. Mono-links are the major products of cross-linking reactions because only a small fraction of residues are in close enough proximity for intermolecular cross-links to occur, and hydrolysis of the reactive esters is faster than the interaction with a second protein reactive group. Mono-links can be easily identified by MS.<sup>20</sup>

The peptides modified with a partially hydrolyzed cross-linker can provide valuable information of the parts on bPrP involved in the binding to mAb3E7. The masses of bPrP peptides modified with DSS-d0/d12 and DSG-d0/d6 as mono-links were calculated (Table 1) and examined in the absence and in the presence of the antibody. The use of isotope-coded cross-linking reagents allows the unambiguous identification of mono-linked peptides based on the mass pattern diagnostic for cross-linker-modified peptides.

The data summarized in Fig. 4 displays the behavior of mono-linked peptides from bPrP alone and in the presence of the antibody. The absolute peak intensities averaged over the elution time of each of the bPrP peptides (Table 2) modified either with DSS-d0/d12 (Fig. 4(A)) or with DSG-d0/d6 (Fig. 4(B)) are plotted. Most of the mono-links were detected in both experiments, except for peptides [150–160] and [153–160] modified by either DSS or DSG, as can be seen in Fig. 4 and in Table 2. A complete fading of the peak intensities corresponding to these peptides was observed after the cross-linking reaction bPrP with the mAb3E7. This indicates that this area on bPrP is not accessible any more to mono-link modification after binding of the antibody. It is most likely due to the formation of an intermolecular cross-link between bPrP and mAb3E7. Another option is that access of the cross-linker to the binding interface is hindered, but this is not supported by

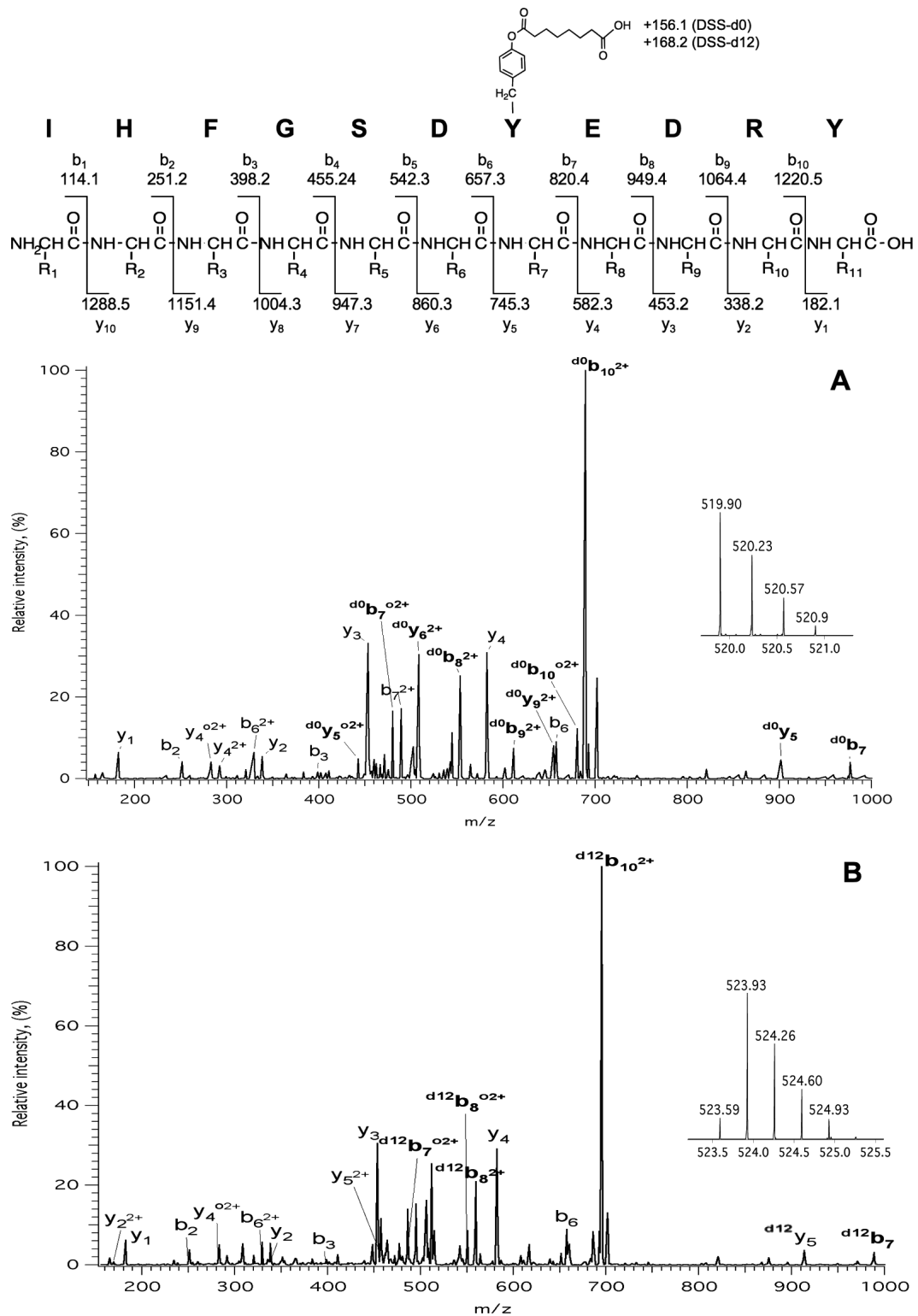


**Figure 5.** FTICR mass spectra of the peptides [150–160] and [230–237] (triply charged signals) analyzed after chymotryptic digestion of the bovine prion protein following different treatments. (A) Peptide [150–160] analyzed before (1) and after treatment of the bovine prion protein with DSS-d0/d12 (2, 3). The bovine prion protein is subjected to the cross-linking reaction in the absence (2) or in the presence (3) of the monoclonal antibody 3E7. (C) Same experiments as A using the cross-linker DSG-d0/d6. (B, D) Same experiments as A and C for the peptide [230–237]. An extracted ion chromatogram for both peptides was used to define the retention time of each peptide of interest. Each spectrum shows an average of all FTICR mass spectra  $\pm 0.5$  min relative to the nondeuterated peptide species. Each spectrum is normalized to the most intense peak.

the data: it would lead to the ‘survival’ of a larger relative intensity of nonmodified [150–160] and [153–160] in the digest, which was not the case. The peak intensities of most peptides were significantly decreased, by about an order of magnitude, in the experiments with the mAb3E7. We interpret this to be due to the higher overall complexity of the cross-linking reaction mixture generated from the digested [mAb3E7·2bPrP] complex and accompanying signal suppression. Although this lowers the confidence for clearly identifying a ‘fading’ of some peptides due to binding with the antibody, the use of isotopically labeled cross-linkers allows clear identification of peptides carrying the cross-linker moiety.

The exact amino acid sequence representing the epitope has been defined earlier as IHFGSD [150–155] by the PEPSCAN method.<sup>34</sup> The PEPSCAN technique uses sets of overlapping synthetic peptides (e.g. hexamers) covering the complete antigen sequence, which are screened with monoclonal antibodies of interest. The part [150–155] of the bPrP sequence is present in two peptides in our study: peptide [150–160] and peptide [153–160]. Among

these, peptide [150–160], which included the amino acid residues IHFGSD, was carefully studied in three independent experiments: digested bPrP without a cross-linker, digested bPrP cross-linked with DSS-d0/d12 and DSG-d0/d6, and digested cross-linked [mAb3E7·2bPrP] complex (Fig. 5). The peaks corresponding to peptide [150–160] modified with DSS-d0/d12 and DSG-d0/d6 (Fig. 5(A), (C)) were compared with those corresponding to another modified peptide [230–237] (Fig. 5(B), (D)), which is not involved in the interaction with the antibody (Fig. 5). The triply charged signals of the peptides were observed. It is clearly visible in Fig. 5(A), (C) that there is total fading of the intensities of the peaks corresponding to peptide [150–160] in the presence of the antibody (trace 3). As a control, the peaks representing the modified peptide [230–237] stay without significant changes (Fig. 5(B), (D)) (trace 3) apart from the overall signal decrease described above. Analysis of peptide [153–160] shows the same behavior as peptide [150–160]. Therefore, it can be concluded that some amino acids in peptide [150–160] must be involved in the interaction with the mAb3E7. The fact that the signal intensities for the modified peptides



**Figure 6.** MS/MS analysis of the peptide [150–160] modified with (A) DSS-d0 and (B) DSS-d12. The sequence of the peptide is shown with masses for the b and y fragments. (A) CID mass spectrum of the precursor ion  $[M + 3H]^{3+}$  at  $m/z$  519.90 (modification with DSS-d0), the ions containing the light mono-link modification are marked with  $d^0$ . (B) CID mass spectrum of the precursor ion  $[M + 3H]^{3+}$  at  $m/z$  523.93 (modification with DSS-d12), the ions with the heavy mono-link modification are marked with  $d^{12}$ . The mass spectra of the parent ions are given in the insets. Fragment ions that are created by an additional loss of water are indicated as  $b^o$  and  $y^o$  ions.

[150–160] and [153–160] completely faded upon complex formation provides indirect but solid information on the epitope region. Thus, our results correlate very well with the results previously obtained with the PEPSCAN method.

### Side chain reactivity towards cross-linkers

Both DSS and DSG are commonly used to target amino acids with primary amines. For example, peptide [111–139] contains three lysine residues in fairly close proximity. In the

mass spectra (data not shown), this peptide appears carrying up to three mono-links, with a statistical distribution of heavy/light isotopomers, albeit with a Mascot score below the identity threshold, most likely due to the length of this peptide. However, the data presented in Table 2 and in Fig. 4 also shows that a number of peptides which were modified with DSS and DSG do not contain primary amino groups. It is hard to believe that only lysine residues in the peptide [111–139] reacted with the employed cross-linking reagents; the bPrP(25–241) sequence does not contain a high number of lysine residues. Moreover, the lysines are unevenly distributed and are absent in the epitope region. For the identified peptides the reactivity of the cross-linking reagents appeared to be mainly directed towards the hydroxyl groups of tyrosine residues and in some cases of serine. For example, peptide [150–160] representing the epitope contains both Tyr and Ser residues, but does not contain any Lys. This reactivity of *N*-hydroxysuccinimide esters was previously noticed by Sinz,<sup>3</sup> and two studies<sup>32,33</sup> were recently published on this topic. To prove the reactivity of DSS and DSG to either the Tyr or the Ser side chain in this peptide, an MS/MS analysis was performed. From the MS/MS data in Fig. 6 it can be clearly seen that fragments  $y_1$  through  $y_4$  were unmodified, and the masses of fragments  $b_7$  through  $b_{10}$  were increased by 156.1 Da for DSS-d0 (Fig. 6(A)) and 168.2 for DSS-d12 (Fig. 6(B)). These results showed that Tyr in position 7 of the peptide IHFGSDYEDRY was modified and apparently led to the complex formation with the antibody. Therefore, the epitope of mAb3E7 was resolved within 4–6 amino acids from the modified side chain of the peptide whose intensity completely faded. The reactivity of *N*-hydroxysuccinimide esters towards Tyr and Ser should always be considered when analyzing the cross-linking products.

### Identification of unknown epitopes

To validate our analytical strategy, we have chosen to work with a system of some complexity, but with a known linear epitope. The results show the capacity of the described approach to identify the peptides that are the part of the epitope. Theoretically, both linear and conformational (discontinuous) epitopes can be studied. To verify this, further investigations are necessary. The generality of this methodology and applicability to other systems should be also explored.

For successful identification of unknown epitopes, it is necessary to know the sequence of at least one of the interaction partners, and to achieve efficient cross-linking of the antigen/antibody complex. A number of parameters have to be optimized for the complex at each step. For instance, the choice of a cross-linking reagent and the reaction conditions, such as molar excess of a cross-linker over the total protein concentration and time of the cross-linking reaction, must be found experimentally, and are expected to differ for systems other than the one investigated here. High-mass MALDI-TOF MS is an advantageous technique for rapid monitoring of the intact cross-linked antigen/antibody complexes and determination of their stoichiometry. In order to reach high efficiency of the cross-linking reactions and to be able to detect and distinguish the cross-linking

products, reagents with different reactivity and design, such as isotopically labeled and trifunctional cross-linkers, can be applied. The availability of high-resolution and high-accuracy instrumentation like FTICR-MS is important for detection of cross-linking products. Another relevant issue when studying unknown epitopes is to reach maximally high (ideally 100%) sequence coverage of an antigen after proteolysis, as well as high sequence coverage with respect to modified peptides with an attached mono-link. This can be achieved by proper choice of the protease or by combination of proteolytic enzymes. A high sequence coverage of modified peptides provides information on cross-linking reactivity and improves the chances to identify an epitope.

### CONCLUSIONS

Development of analytical methods for rapid epitope determination is crucial in modern immunology, immunochemistry and immunomedicine. In the present work an analytical strategy for epitope mapping by chemical cross-linking, high-mass MALDI-TOF MS and nano-LC-ESI-FTICR MS was described. The epitope on bPrP(25–241) recognized by a monoclonal antibody 3E7 was identified; the results are in excellent agreement with PEPSCAN data. This approach is shown to be a promising analytical tool to rapidly determine the epitope in large antigen/antibody complexes. It is conceptually simple, and only picomole quantities of proteins are needed.

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