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RESEARCH ARTICLE

Epitope Ligand Binding Sites of Blood Group Oligosaccharides in Lectins Revealed by Pressure-Assisted Proteolytic Excision Affinity Mass Spectrometry

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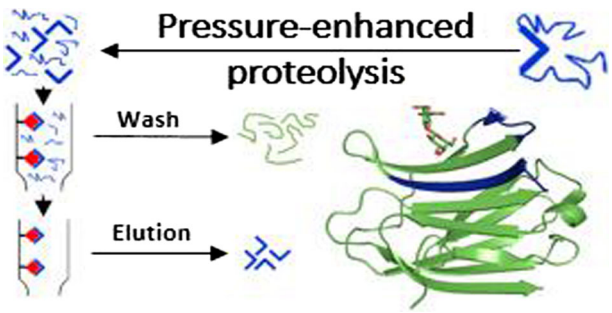

Abstract. Affinity mass spectrometry using selective proteolytic excision and extraction combined with MALDI and ESI mass spectrometry has been applied to the identification of epitope binding sites of lactose, GalNac, and blood group oligosaccharides in two blood group-specific lectins, human galectin-3 and glycine max lectin. The epitope peptides identified comprise all essential amino acids involved in carbohydrate recognition, in complete agreement with available X-ray structures. Tryptic and chymotryptic digestion of lectins for proteolytic extraction/excision-MS was substantially improved by pressure-enhanced digestion using an automated Barocycler procedure (40 kpsi). Both previously established immobilization on affinity microcolumns using divinyl sulfone and coupling of a specific peptide glycoprobe to the gold surface of a biosensor chip were successfully employed for proteolytic excision and extraction of carbohydrate epitopes and affinity measurements. The identified epitope peptides could be differentiated according to the carbohydrate employed, thus demonstrating the specificity of the mass spectrometric approach. The specificities of the epitope ligands for individual carbohydrates were further ascertained by affinity studies using synthetic peptide ligands with immobilized carbohydrates. Binding affinities of the synthetic ligand peptides to lactose, in comparison to the intact full-length lectins, were determined by surface acoustic wave (SAW) biosensor analysis and provided micromolar K_D values for the intact lectins, in agreement with results of previous ITC and SPR studies. Binding affinities of the epitope peptides were approximately two orders of magnitude lower, consistent with their smaller size and assembled arrangement in the carbohydrate recognition domains.

Keywords: Mass spectrometry, Human galectin-3, Glycine max lectin, Blood group oligosaccharides, CRD, Recognition sites, Proteolytic excision, proteolytic extraction, Ligand epitope peptides, SAW-biosensor analysis

AbbreviationsCRDCarbohydrate recognition domain; AoaAminooxyacetic acid; DVSDivinyl sulfone; GalNAcAcétylgalactosamine; PBSPhosphate-buffered saline; DTTDithiothreitol; SAMSelf-assembled monolayer; SAWSurface acoustic waves

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Introduction

Interactions of carbohydrates with proteins such as lectins belong to the most important types of biological processes, e.g., in cellular recognition, intracellular regulation pathways, and immunological reactions. Complete structures of oligo- and polysaccharide complexes with lectins and antibodies have been determined in a few cases both by X-ray crystallography and by nuclear magnetic resonance spectroscopy. Crystal structures of complexes with glycans have been defined for antibodies, plant and animal lectins, bacterial toxins, and carbohydrate-binding enzymes. However, x-ray crystallography approaches are limited by the notorious difficulties in crystallization of protein-glycan complexes at sufficiently high resolution, as well as by the high requirements of relatively large amounts and highly purified samples [1].

We have recently developed a new approach for the molecular identification of carbohydrate recognition epitopes in lectins with high specificity, sensitivity, and low requirements of sample purity [2, 3], using a combination of proteolytic digestion and mass spectrometry. In a first variant of the analytical approach termed proteolytic excision [4–7], the carbohydrate is immobilized on an affinity column and the carbohydrate-binding protein (lectin, antibody, etc.) added to form a complex which is then subjected to proteolytic digestion. In a second variant, proteolytic extraction, the protein is first subjected to proteolytic digestion and the mixture of peptide fragments presented to the immobilized carbohydrate. After washing away the non-binding peptides, the remaining affinity-bound peptide ligand fragments are eluted and analyzed by mass spectrometry (Fig. 1a). This approach has been generally termed "Carbohydrate Recognition Domain Excision ("CREDEX") [3].

In the CREDEX approach, the proteolytic step has been often a major problem, since intact lectins are highly stable to enzymatic hydrolysis and require time-consuming proteolytic digestion. Here, we demonstrate the application of high-pressure proteolytic digestion as an efficient tool for the mass spectrometric identification of carbohydrate-binding peptides in animal and plant lectins. In the final elution step, competitive elution with a carbohydrate solution is generally suitable for specific isolation of ligand epitopes; however, it requires high concentrations. Here, we show that a wide range of organic-aqueous eluent mixtures are feasible as substitutes for elution [2, 3].

In this study, we have applied the CREDEX-MS method using pressure-enhanced proteolysis to the identification of ligand epitopes of blood group oligosaccharides to human galectin-3 (hGal-3) and glycine max lectin (Soybean Agglutinin, SBA). The results show specific epitope peptides residing in the structures of lectins, which provide a molecular differentiation of oligosaccharide epitopes that should be applicable to a blood group determination assay.

Experimental

Lectins and Proteolytic Enzymes

Recombinant human galectin-3 was obtained from Carbosynth, Compton (UK). Hgal-3 was purified from extracts of bacteria by affinity chromatography on lactosylated Sepharose 4B and analyzed for purity by one- and two-dimensional gel electrophoresis and gel filtration as previously described [2, 3]. It was checked for activity by solid-phase and cell-based assays [3, 8]. Glycine max lectin isolated from soy bean was purchased from Medicago AB (Uppsala, Sweden) and was purified by affinity chromatography. Trypsin and chymotrypsin, sequencing grade, were obtained from Promega (Mannheim, Germany).

Carbohydrate Ligands

GalNAc and β-lactose were obtained from Sigma Aldrich (Steinheim, Germany). Blood group A- and B-trisaccharides and -tetrasaccharide were obtained from Carbosynth Ltd. (Compton, UK). The blood group A- and B-trisaccharides were conjugated and tested for bioactivity as described [3], then conjugated to Sepharose beads to establish the affinity matrix for galectin binding in the same manner, as carried out for lactose [2].

Immobilized Carbohydrate Columns

Two hundred microliters drained Sepharose 4B was washed with 1 mL 0.5 M Na_2CO_3 pH 11 and drained. The Sepharose was resuspended in 300 mL of 0.5 M Na_2CO_3 and 40 µL divinyl sulfone (DVS) was added dropwise. The suspension was mixed end-over-end in a 90° rotary sample mixer for 70 min at 25 °C in the dark. The matrix was drained and washed with 2 mL 0.5 M Na_2CO_3 pH 11 and resuspended in 200 µL of 1% carbohydrate solution in 0.5 M Na_2CO_3 pH 10 and mixed for 18 h at 25 °C. The affinity matrix was then washed with 2 mL 0.5 M Na_2CO_3 pH 10. Blocking of unreacted vinyl groups was carried out by incubation with 1 M ethanolamine pH 8.5 for 1 h at 25 °C. Next, the matrix was washed sequentially with 0.1 M ammonium acetate containing 0.5 M NaCl, pH 4, and 0.1 M Tris-HCl/0.5 M NaCl, pH 8. Finally, the matrix was equilibrated in PBS buffer (25 mM Na_2HPO4/20 mM NaCl, pH 7.5) for hGal-3 binding or HEPES buffer (10 mM HEPES, 25 mM $NaCl_2$, 5 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4) for SBA binding and stored at 4 °C.

For control experiments, a Sepharose blank was prepared by activating the matrix with DVS as described above. The matrix was then treated with 1 M ethanolamine for 18 h at 25 °C and washed with buffers using the same sequence as described above.

Proteolytic Digestion

Due to the high resistance of lectins to enzymatic hydrolysis, SBA was first denatured at 100 °C for 25 min prior to digestion.

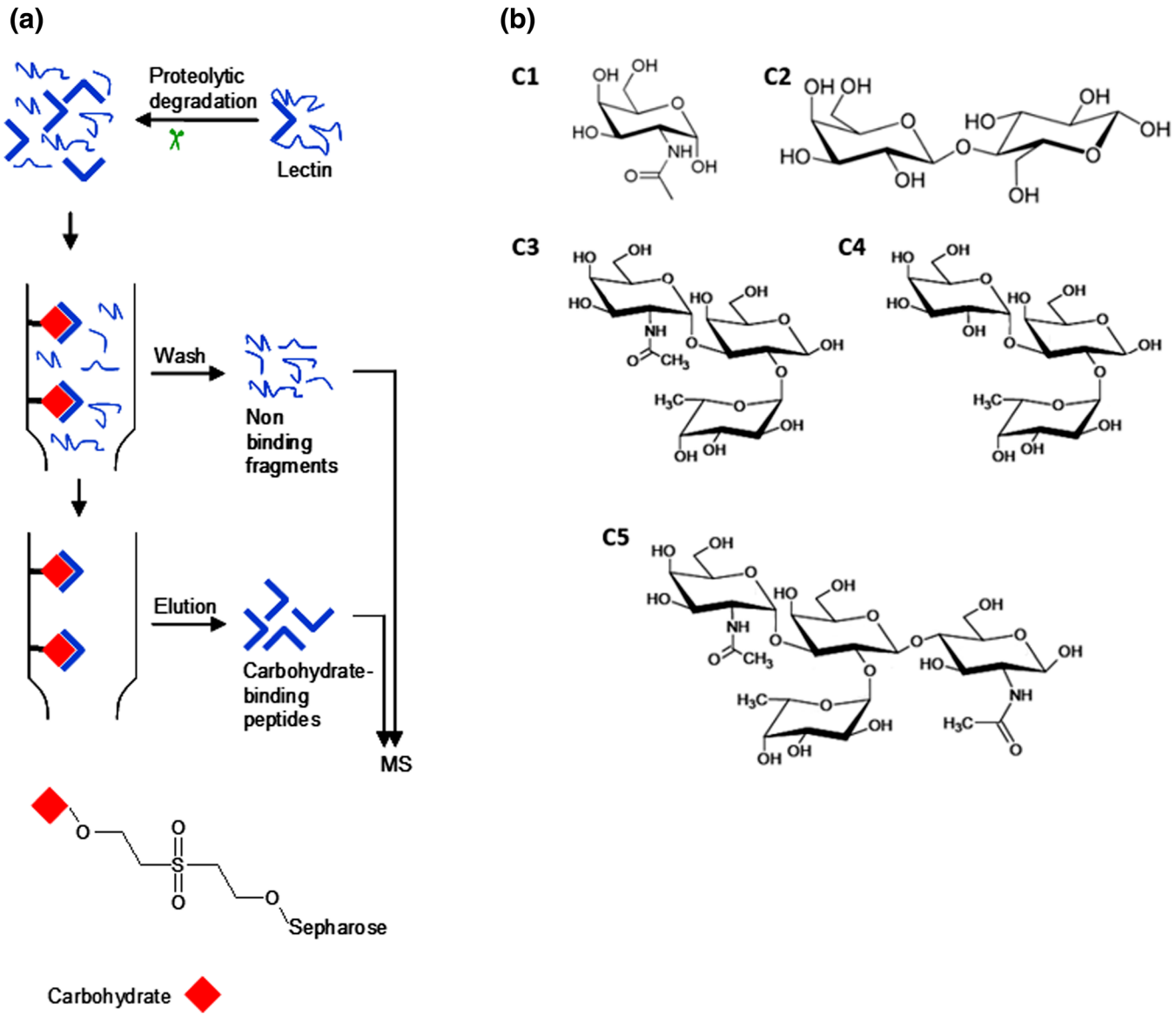

Figure 1. **(a)** Proteolytic extraction mass spectrometric approach for identifying carbohydrate-binding structures in proteins. The lectin is first digested in solution and the resulting fragments are bound on a column with immobilized carbohydrate. The non-binding peptides are removed by washing and the binding fragments are subsequently eluted. **(b)** Chemical structures of the carbohydrate ligands investigated: (C1) *N*-Acétylgalactosamine (2-acétamido-2-désoxy-D-galactopyranose), (C2) β-lactose (β-D-galactopyranosyl-(1-4)-β-D-glucose), (C3) blood group A-trisaccharide (α-L-Fuc-(1-2)-[α-D-GalNAc-(1-3)]-β-D-Gal), (C4) blood group B-trisaccharide (α-L-Fuc-(1-2)-[α-D-Gal-(1-3)]-β-D-Gal), (C5) blood group A-tetrasaccharide type 2 (α-D-GalNAc-(1-3)-[α-L-Fuc-(1-2)]-β-D-Gal-(1-4)-β-D-GlcNAc)

A mixture of trypsin and chymotrypsin (enzyme/substrate ratios 1:20 and 1:40, respectively) in Tris-buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0) was used for SBA, while hGal-3 was digested using trypsin (enzyme/substrate ratio, 1:20) in phosphate-buffered saline (PBS, 25 mM, pH 7.5) with 1 g/L dithiothreitol (DTT). Proteolytic digestion was then performed at high pressure (50 s at 40 kpsi, 10 s at atmospheric pressure) for 120 cycles at 37 °C using a Barocycler 2320EXT (Pressure Bioscience, Boston, MA, USA). A 1-µL aliquot sample from the digestion mixture was then subjected to MALDI-MS analysis.

Proteolytic Extraction-Mass Spectrometry

The SBA and hGal-3 digest mixtures were diluted 1:2, respectively, using HEPES buffer and PBS prior to the addition onto the affinity column containing immobilized carbohydrates. 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 four times 400 µL HEPES for SBA and PBS for hGal-3, and the washing fraction was analyzed by MALDI-MS. The peptides bound to the immobilized carbohydrate were eluted under shaking with 400 µL ACN/0.1% TFA (2:1) at 37 °C for 15 min, and the procedure repeated twice. The elution fractions were then combined, concentrated, and analyzed by MALDI-MS.

Proteolytic Excision-Mass Spectrometry

A solution of 20 µg SBA or hGal-3 in respectively 200 µL HEPES or PBS buffer, pH 7.5, was added to 200 µL affinity matrix and allowed to bind for 12 h at 37 °C. Any free (unbound) lectin was washed out with 2 × 400 µL binding buffer (with 1 g/L DTT) and the washing fractions were analyzed by MALDI-MS. The binding buffer was then switched to Tris-buffer (with 5 mM $MnCl_2$ added to allow carbohydrates to interact with SBA) and proteolytic digestion of bound lectin was performed by application to high-pressure cycles (30 kpsi for 50 s, atmospheric pressure for 10 s, 50 °C, 90 cycles) with a mixture of trypsin and chymotrypsin (1:20 and 1:30). Following digestion, the supernatant was analyzed by MALDI-MS. Free (unbound) peptides were washed out with 4 × 400 µL binding buffer and the washing fractions analyzed by MALDI-MS. The affinity-bound peptides were then eluted with two times 200 µL ACN/0.1% TFA 2:1 at 37 °C for 15 min under shaking.

Peptide Synthesis

Based on the identification of lactose and carbohydrate-binding site peptides [2], three human galectin-3 peptides were synthesized by SPPS: hGal-3(152–162), GNDVAFHFNPR (**1**); hGal-3(177–183), LDNNWGR (**2**); hGal-3(130–144), MLITILGTVKPNANR (**3**). The lactose carrier peptide, *N*-Me-Aoa-Gly-Phe-Lys-Lys-Gly-COOH was synthesized and lactosylated as previously described [2, 3]. All peptide syntheses were carried out on a NovaSyn TGR resin according to the Fmoc/tBu strategy [9, 10] using a semi-automated peptide synthesizer (Intavis EPS 221; Köln, Germany). Preparative RP-HPLC purification of the synthetic peptides were carried out on a Knauer HPLC system (Bad Homburg, Germany) using a GROM-SIL 120 ODS-4 SE HPLC column (Grom, Herrenberg-Kayh, Germany). HPLC analyses were monitored by UV detection at 220 nm. Sequence and homogeneities of all peptides were confirmed by ESI-MS.

Affinity Mass Spectrometry of Galectin-Derived Peptides

For each of the synthetic peptides (s. Table 1), a solution of 30 µg peptide or lectin in PBS pH 7.5 was added over the affinity column with carbohydrates (lactose, A-Tri, or B-Tri) immobilized on DVS-activated Sepharose. After 12 h shaking at 37 °C, the supernatant was analyzed by MALDI-MS. The columns were then washed with binding solvent until the washing fractions did not provide any signals by MALDI-MS. Elution with lactose-containing buffer was then performed twice with 400 µL 0.3 M lactose in PBS (pH 7.5) while shaking at 37 °C for 15 min. A surrogate elution system containing acetonitrile was employed on an alternative set of samples, by adding 400 µL ACN/0.1% TFA 2:1 onto the Sepharose slurry and shaking at 37 °C for 15 min. This procedure was carried out twice, and each time the eluate was collected.

Mass Spectrometry

ESI mass spectra were obtained with a Bruker (Bruker Daltonik, Bremen, Germany) Esquire 3000+ and a Bruker HCT ion trap mass spectrometer. A Bruker Autoflex Smartbeam mass spectrometer equipped with external fully automated X-Y target stage MALDI source with pulsed collision gas was employed for MALDI-MS analyses. The pulsed nitrogen laser was operated at 337 nm and ions generated by 10 laser shots were accumulated for 0.5–1 s at 30 V and extracted at 15 V. Mass spectra were obtained using 10 laser shots for each scan and accumulating 30–60 scans. The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in 1 mL acetonitrile/0.1% TFA in water (2:1 *v*/*v*). A 0.5-µL aliquot of matrix solution was deposited on the MALDI target, mixed with 0.5 µL of sample solution and allowed to dry in ambient air. External calibration was performed using the monoisotopic masses of singly protonated ions of human angiotensins I and II, bradykinin, neurotensin, bovine insulin ß-chain (oxidized), and bovine insulin.

Aliquots of 1 µL of the sample solution and the saturated matrix solution were mixed on the stainless steel MALDI target and allowed to dry. Acquisition of spectra was carried out at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV.

Determination of Affinity Binding Constants

Binding studies of synthetic peptides and full-length galectins and glycine max lectin with lactose and GalNAc were performed with a surface acoustic wave (SAW) biosensor (K5-Ssens; SAW-Instruments, Bonn, Germany [11]; Nanotemper GmbH, München, Germany).

A monolayer (SAM) of 16-mercaptohexadecanoic acid was formed on the surface of the chip, as previously described [12]. The GalNAc-lactosyl-glycoprobe was immobilized on the SAM by carboxyl-group activation with a 1:1 mixture (*v*/*v*) of 200 mM (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) [12]. A 10-µM aqueous glycoprobe solution (pH 6.5) was used for immobilization, followed by capping of unreacted carboxyl groups with 1 M ethanolamine, pH 8.5. After changing the running buffer to HEPES (pH 7.4) for SBA or PBS (pH 7.5) for hGal-3 and synthetic peptides, 150 µL solutions of increasing concentrations (0.1–1 mM PBS) of synthetic human lectin peptides were injected on the chip with the immobilized glycoprobe at a flow rate of 30 µL min^{-1}. Intact lectins were injected at concentrations of 0.5–10 µM in buffer in their corresponding buffer at the same flow rate. After each injection, the bound

Table 1. Summary of K_D determinations between hGal-3 and synthetic hGal-3 peptides against lactose, and full-length SBA against GalNAc

No.	Peptide/protein	Immobilized carbohydrate	K_D (mM)	R^2
1	hGal-3(152–162)	Lactose	11.7	0.97
2	hGal-3(177–183)	Lactose	2.4	0.99
3	hGal-3(130–144)	Lactose	n.a.	n.a.
4	hGal-3 full length	Lactose	4.8×10^{-3}	0.98
5	SBA full length	GalNac	25.5×10^{-3}	0.99

peptide was left to dissociate for 5 min and the chip surface was regenerated with 60% ACN, pH 6.5. After 5 min equilibration time the next injection was allowed. After fitting the recorded data according to the theoretical 1:1 Langmuir binding model, the extracted observed rate constants ($k_{obs} = k_{off} + C_{analyte} \times k_{on}$) were plotted versus the analyte concentrations. Linear regression of the data points yielded the association (k_{on}) and dissociation (k_{off}) rate constants that were used to obtain the equilibrium dissociation constant $K_D = k_{off} \times k_{on}^{-1}$. The obtained K_D values are summarized in Table 1. A negative control for determination of unspecific binding was performed by injecting a solution of 0.5–20 µM myoglobin in PBS at identical conditions as described above.

Results and Discussion

Primary Structure Characterization of Lectins by Proteolytic Peptide Mapping

As a first step in the identification of carbohydrate-binding sites, the primary structures of the investigated lectins were characterized by proteolytic peptide mapping and mass spectrometry, particularly to establish the conditions for the proteolytic excision and extraction, and formation of regular, reproducible peptide fragment mixtures. Native SBA has been previously reported to be highly resistant to enzymatic hydrolysis, requiring heat treatment and multiple proteases for complete digestion [13]. Cycles of high pressure during proteolytic digestion uses alternating high hydrostatic pressure to facilitate thermodynamic perturbation of molecular interactions and has been shown to increase hydrolysis speed and recovery and improve sequence coverage [14, 15]. The use of pressure-enhanced proteolytic digestion provided a complete lectin digestion within 1–2 h, whereas 12 h were necessary to obtain comparable digestion results using a standard in-solution digestion protocol at 37 °C and atmospheric pressure. A comparison of the MALDI-MS data of SBA for both digestion methods after 2 h showed the incomplete digestion of the in-solution procedure with a calculated sequence coverage of 32% compared to the 90% sequence coverage obtained with the high-pressure digestion at 40 kpsi (Fig. 2). Nearly full sequence coverage can be obtained with proteolytic peptide mapping on both lectins, except for small sequences of 1–4 amino acids ($m/z < 600$) and the Asp-75 glycosylated peptide (71–75) of glycine max lectin (Fig. 2; Table S1, Supporting Information). SBA is known to form C-terminal truncated isolectin structures with 246 amino acids, which explains the cleavage at Ser246 [16]. Digestion times and enzymes/substrate ratios were optimized as described in the "Experimental" section and digestion yields were estimated for all lectins by 1D gel electrophoresis.

Epitope Ligand Identification and Differentiation of Blood Group Oligosaccharides in Lectins

Carbohydrates with different structural features (lactose; GalNAc; blood group A- and B-trisaccharides; blood group A-tetrasaccharide) were investigated as ligands (s. Fig. 1b). SBA shows high affinities to GalNAc and galactose moieties [17], which prompted us to study interactions with GalNac, blood group A- and B-trisaccharides. MALDI-MS analyses of the elution fractions upon proteolytic excision and extraction from the affinity columns performed with immobilized GalNAc showed ions corresponding to 3 specific peptide sequences (87–107) (**1**), (129–147) (**2**), and (206–224) (**3**) (Fig. 3a). The identical epitope ligands were found in the elution fractions from A- and B-trisaccharides, with an additional specific peptide sequence (108–125) (**4**) (Fig. 3b, c). The epitope peptides (**1**), (**2**), and (**3**) identified in the elution fractions contain the amino acid residues Asp-88, Gly-106, Asn-130, and Asp-215 in direct contact with the carbohydrate ligand, in complete agreement with the X-ray crystal structure data (s. Fig. 6a). In the crystal structure, Rao et al. reported that Asp-88 forms a bidentate hydrogen bond with C3-0H and C4-0H hydroxyl groups, while the nitrogen group ND2 of Asn-130 and of Gly-106 form hydrogen bonds with C2-0H and C3-0H hydroxyl groups, respectively, and the 4-0H hydroxyl group forms another hydrogen bond with the backbone-NH of Leu-214 [18]. The fourth peptide domain (**4**) identified in the elution fractions of A- and B-trisaccharide include the loop (112–119), where electron density has been found to be weak, and is located close to the sugar binding site [18] (s. Fig. 6a). The presence of (**4**) in the elution fraction can therefore be explained by an interaction between the branched oligosaccharides and the peptide loop.

An identical set of experiments provided the identification of the carbohydrate recognition domains of hGal-3. MALDI-MS analyses of the elution fractions upon proteolytic excision and extraction from the affinity columns performed with immobilized lactose and B-trisaccharide, containing affinity-bound hGal-3 peptides, showed only two molecular ions corresponding to peptides (152–162) (**1**) and (177–183) (**2**). From the corresponding experiments with immobilized A-tri- and A-tetrasaccharides, the identical peptides (**1**) and (**2**) and a specific additional third peptide (130–144) (**3**) were identified (Figs. 4a,

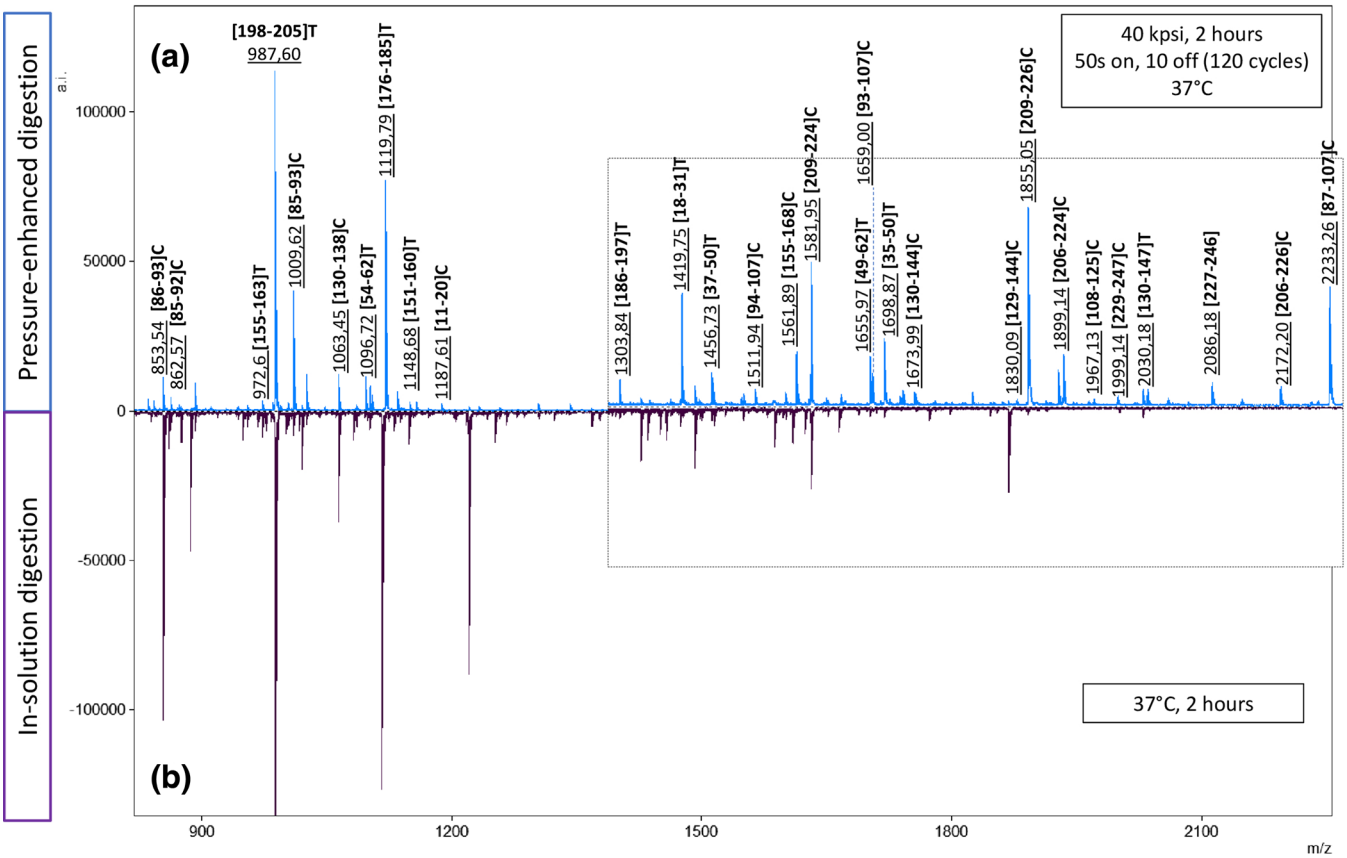

Figure 2. Comparison of MALDI-TOF mass spectra of SBA proteolytic digestion after 120 cycles of 50 s at 40 kpsi and 10 s at atmospheric pressure and 37 °C **(a)** and standard proteolytic digestion at atmospheric pressure and 37 °C **(b)**. Digestion procedures were performed with a mixture of trypsin and chymotrypsin (enzyme/substrate ratios 1:20 and 1:40, respectively) in Tris-buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0). All peptide fragments produced singly charged ions and are labeled T for tryptic and C for chymotryptic cleavage products. In addition, peaks corresponding to sodium and potassium adducts ($[M + Na^+]^+$, $[M + K^+]^+$) were observed in all spectra. The region with $m/z > 1300$ Da has been magnified

b and 6a). These results revealed that the human galectin-3 binding region for lactose and B-Tri is formed by amino acids from the domains (152–162) and (177–183) (s. Fig. 4a). The peptides (**1**) and (**2**) comprise the key amino acids that are in direct contact with the ligand, in agreement with previous results by X-Ray crystallography [19–21]. The majority of the interactions are formed between the side-chains of His158, Asn60, and Arg162 from peptide (**1**) and the galactose subunit. In peptide (**2**), the residue W-183 participates through its planar side-chain in a CH-π interaction with the B face of the galactose ring. The side-chain of the W-183 residue is not only involved in binding of the galactose ring but also determines its orientation.

In addition to the peptides (152–162) and (177–183), the recognition sites for A-tri and A-tetra include the peptide (130–144) (Figs. 4b and 6b). The presence of this peptide in the elution fraction is well explained by the Arg-144 residue which is positioned to interact with other saccharide residues linked to the galactose O-3 group [20–23]. In the case of A-tri- and A-tetra-saccharide, the O-3-linked residue is α-D-GalNAc. It is noteworthy that B-tri also has a subunit linked to the galactose O-3, α-D-Gal; however, the third peptide, (130–144) was not identified as a binding site for B-tri. These results suggest that peptide **3** (130–144) is specific for α-D-GalNAc and does not bind to α-D-Gal. To support this conclusion, affinity-MS experiments were performed with a synthetic version of peptide **3**, prepared by solid-phase peptide synthesis. In agreement with the CREDEX-MS results, the peptide (130–144) indeed was ascertained to bind only to A-tri- and A-tetrasaccharide, but did not bind to B-trisaccharide or to lactose.

Elution Solvents for Dissociation of Carbohydrate--Peptide Complexes

Elution of the affinity-bound peptides can be performed with a concentrated solution of the identical carbohydrate immobilized on the affinity column; the carbohydrate in solution thereby displacing the affinity-bound peptides from the immobilized carbohydrate in a competitive manner. A different carbohydrate may be used for elution if the affinity of the protein or peptide is comparable to the immobilized carbohydrate. To prevent formation of cysteine- containing peptide dimers through disulfide bonds, DTT (1 g/L) can be added to the eluent without adverse effects. For the elution of affinity-

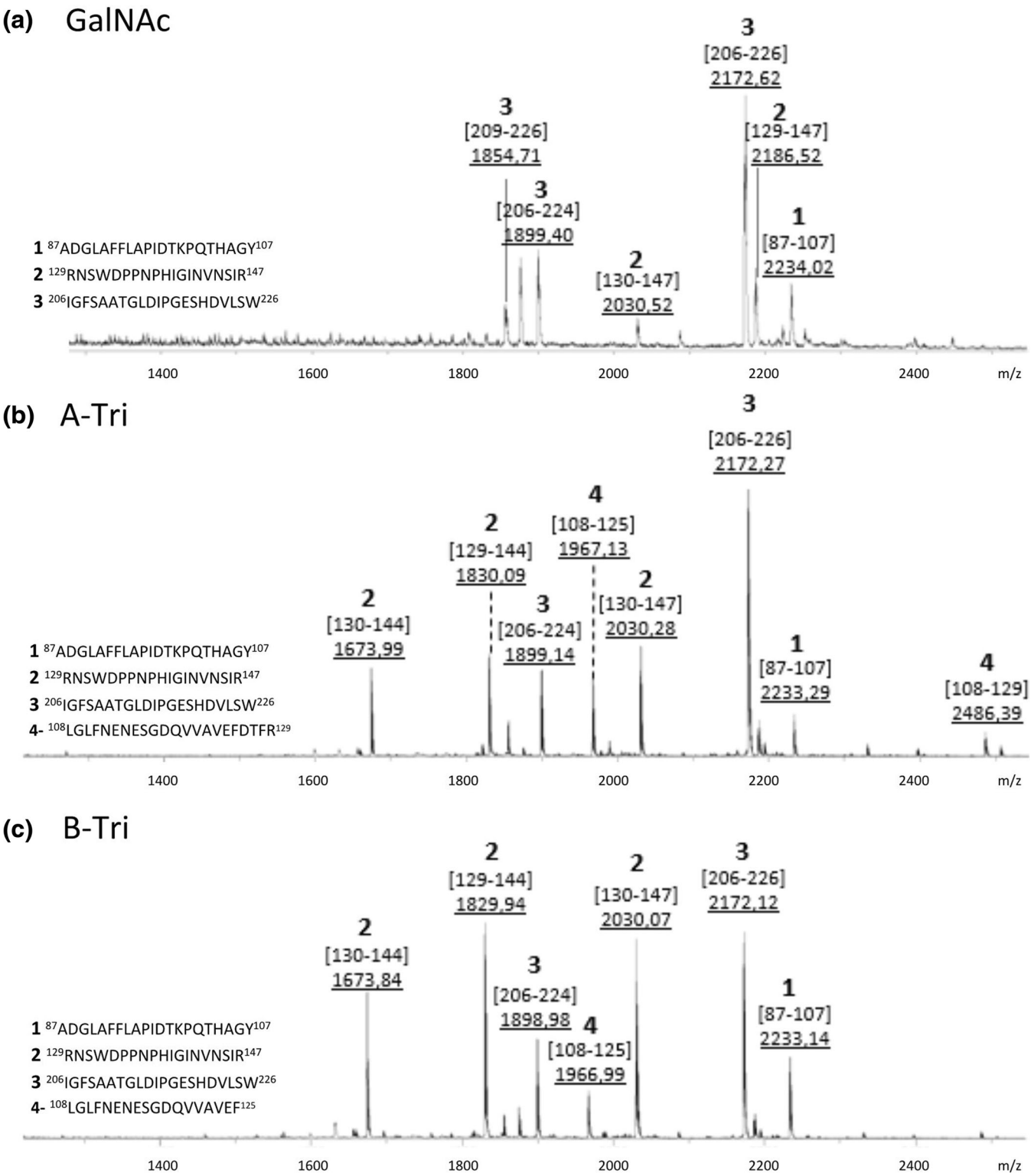

Figure 3. MALDI-TOF mass spectra of elution fractions from proteolytic excision of glycine max lectin form GalNAc **(a)**, A-tri **(b)**, and B-tri **(c)** affinity columns. Peptides (1) correspond to SBA[87–107]; peptides (2) correspond to SBA[129–144], SBA[130–144], and SBA[130–147]; peptides (3) correspond to SBA[206–224] and SBA[206–226]; and peptides (4) correspond to SBA[108–125] and SBA[108–129]. In addition, peaks corresponding to sodium and potassium adducts ($[M + Na^+]^+$, $[M + K^+]^+$) were observed in all spectra

bound galectin-3 peptides from 200 µL matrix with immobilized lactose, the column was shaken with 400 µL 0.3 M lactose in 25 mM phosphate buffer with 25 mM NaCl, pH 7.5 at 37 °C for 15 min, and this procedure repeated twice.

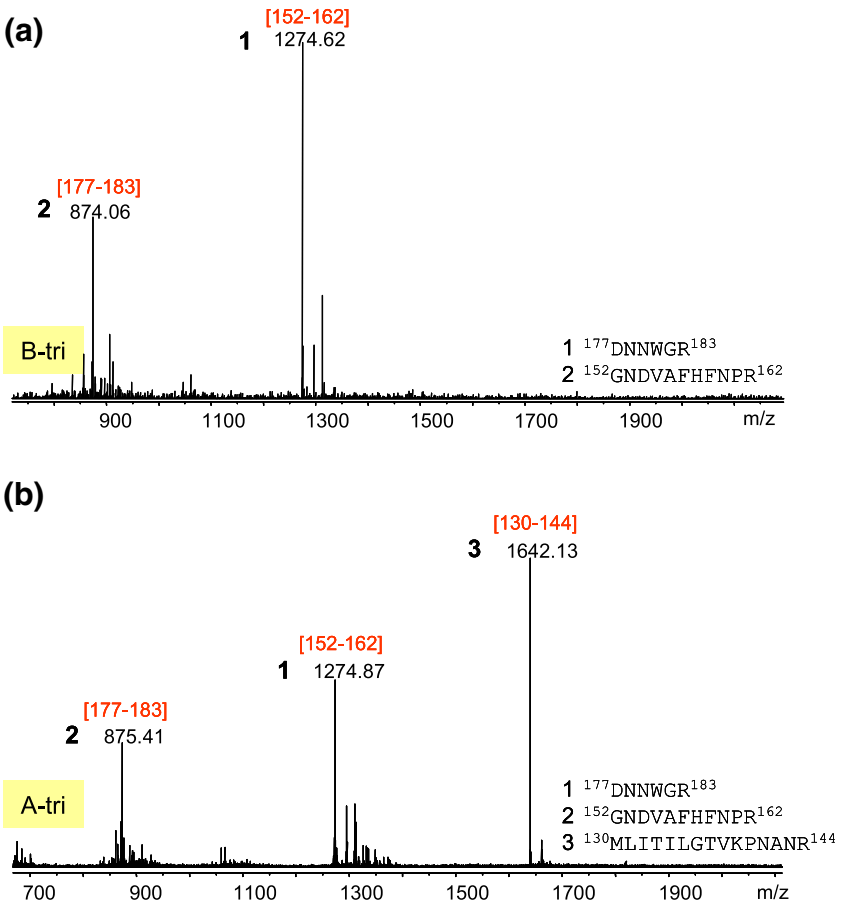

Figure 4. MALDI-TOF mass spectra of elution fractions from proteolytic excision of human galectin-3 form B-tri and A-tri columns. **(a)** Peptides hGal-3(177–183) and hGal-3(152–162) were identified from the lactose and B-tri columns. **(b)** Peptides hGal-3(177–183), hGal-3(152–162), and hGal-3(130–144) were identified from the A-tri and B-tri columns. Additional peaks corresponding to sodium and potassium adducts ($[M + Na^+]^+$, $[M + K^+]^+$) were observed in all spectra

Due to the high concentration of carbohydrate required for quantitative elution of affinity-bound peptides, sample cleaning prior to MS analysis would be necessary even for HPLC-MS. Since the cleaning step may lead to incomplete peptide recovery, elution with aqueous-organic solvents would be advantageous. Several combinations of TFA, HCl, acetic acid, THF, ACN, and methanol were initially tested with varying results.

The most successful solvent systems were based on acetonitrile, particularly ACN/0.1% TFA, 2:1 (*v*/*v*), pH 3, or 60–80% ACN in water, pH 6. Elution was performed in the same manner as for competitive elution, by shaking with 400 µL eluent for 15 min and repeating twice this procedure. Since acetonitrile-containing solutions had a dehydrating effect on agarose-based affinity matrices depending on the percentage of ACN, the matrix was equilibrated in the same buffer used for protein binding immediately after the elution step. A 100% ACN solution did not recover the bound peptides and completely dehydrated and irreversibly damaged the affinity matrix. Other elution systems such as 500 mM acetic acid or 5–10% ACN in water did not perform well, and also solutions based on non-volatile substances such as glycine or chaotropic agents were found unsuitable. Elution from columns with immobilized glycans containing sialic acid was effective at acidic pH, but this rendered the column inactive for subsequent applications due to cleavage of the sialic acid; however, this effect could be completely prevented by using 70% ACN in 25 mM PBS, pH 7.5.

The above ACN-containing elution solvents were initially developed with synthetic lectin peptides but proved equally effective for full-length lectins. The efficacy of the organic eluent solvents on full-length lectins was established using 1D-SDS-PAGE which allowed easy evaluation of the intensity of gel bands of supernatant, washing, and elution fractions.

Affinity Mass Spectrometric and Biosensor Characterization of Lectin-Derived Peptides

To evaluate the binding properties of the synthetic peptide epitopes, peptides were added over Sepharose affinity columns with the immobilized carbohydrate; following incubation, the columns were washed until complete absence of ions detectable by mass spectrometry. Elution of affinity-bound peptides was performed with the acetonitrile-based solvents described above, and elution fractions analyzed by MALDI-TOF-MS. Due to the complete lack of peptide ions in the washing fractions, ion signals in the elution fraction corresponding to

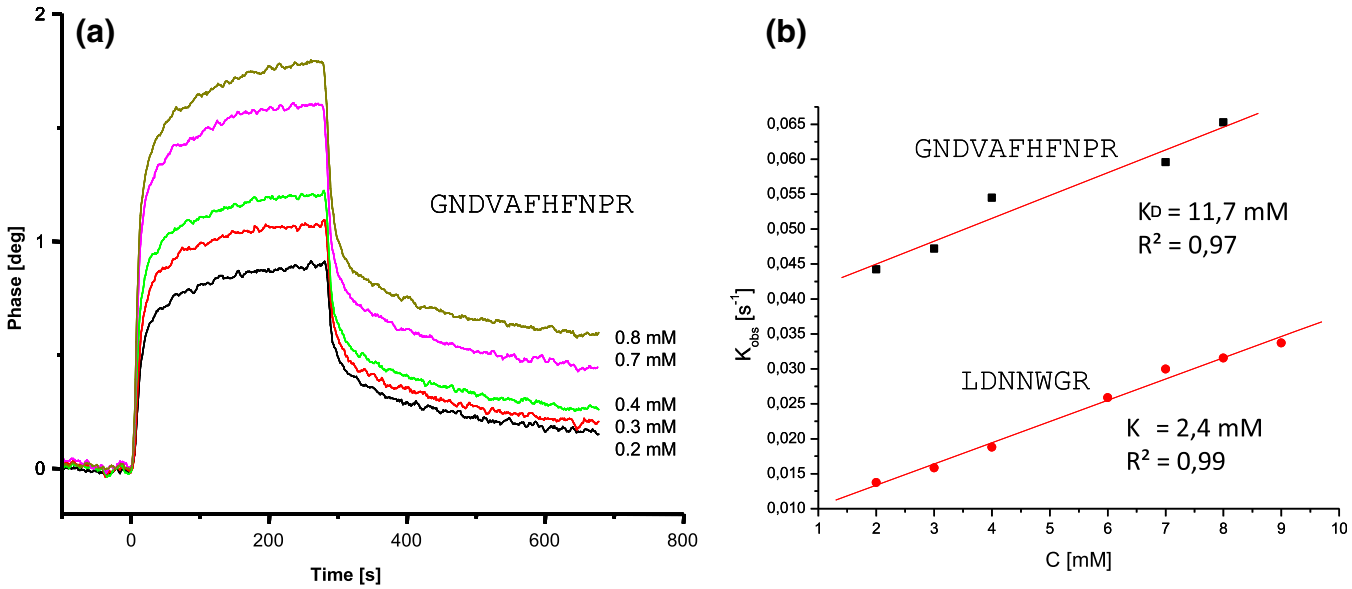

Figure 5. K_D determination for the complex between synthetic human galectin-3 peptides and lactose. **(a)** Fitted association and dissociation curves of hGal-3(152–162) in a series of concentrations (0.2–0.8 mM) with the immobilized lactosyl-glycoprobe. **(b)** The plot of k_{obs} values versus peptide concentrations provided a K_D of 11.7 mM for hGal-3(152–162) and 2.4 mM for hGal-3(177–183)

the peptide ascertain the affinity of the peptides to the carbohydrate. In agreement with the results obtained by CREDEX-MS, the peptides hGal-3(152–162) (**1**), hGal-3(177–183) (**2**), and hGal-3(130–144) (**3**) all showed affinity towards lactose, A-trisaccharide, and B-trisaccharide. Three different sets of controls were performed for all peptides using Sepharose-coupled maltose, Sepharose-coupled sucrose, and unmodified Sepharose. None of the peptides exhibited affinity towards maltose, sucrose, or to the unmodified Sepharose (data not shown).

Dissociation constants K_D were determined with the SAW biosensor for the full-length galectin and SBA in complexes

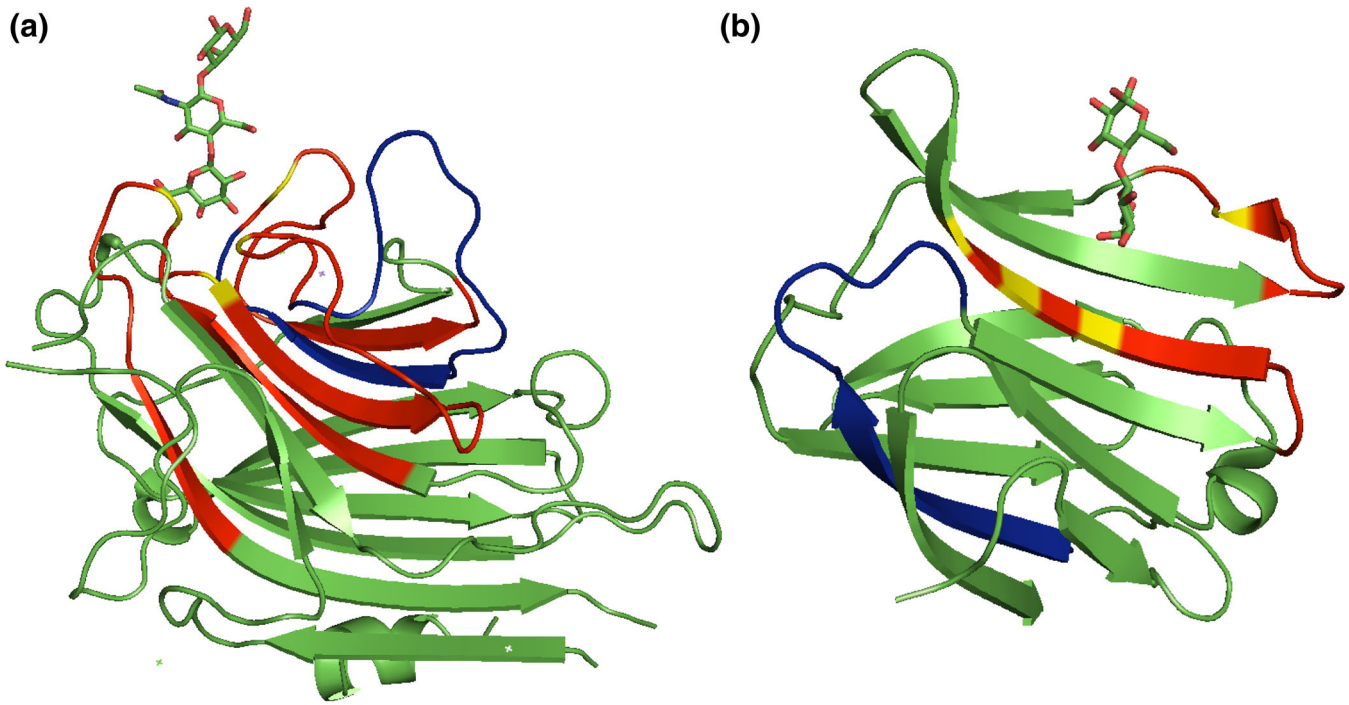

Figure 6. **(a)** X-ray crystal structure of SBA in complex with a biantennary blood group antigen analog (PDB entry 1G9F) showing SBA[87–107], SBA[129–147], and SBA[206–226] in red and MS-identified peptides specific for A-tri and B-tri, SBA[108–128], in blue. Amino acids known to be in direct contact with the carbohydrate are highlighted in yellow. **(b)** X-ray crystal structure of human galectin-3 CRD in complex with lactose (PDB entry 3ZSJ) showing hGal-3(152–162) and hGal-3(152–162) in red and MS-identified peptides specific for A-tri and A-tetra, hGal-3(130–144), in blue. Amino acids known from the complex with lactose to be in direct contact with the carbohydrate are highlighted in yellow

with lactose and GalNAc, respectively. The K_D values were in the low micromolar range, in agreement with the values reported for galectins using SPR [23, 24] and ITC [25]. The synthetic peptide epitopes had significantly lower affinities for lactose (approximately 1 to 14 mM) than the full-length proteins, which is well explained as the small peptides represent just a section of the complete CRD structure, respectively. Repetitive measurements showed a reproducibility of 2 to 5% depending the on the level of affinities measured. The two human peptides hGal-3(177–183) and hGal-3(152–162) showed K_D values of 2.4 and 11.7 mM, respectively (Figs. 5 and 6). Shortening of the sequence (152–162) to (157–163) led only to a slight decrease in affinity (K_D 13.8 mM), while sequence prolongation to (157–175) to include Val171 and Asn174 residues provided a 20-fold increase in affinity (K_D 0.7 mM).

Conclusions

The combination of pressure-assisted enzymatic hydrolysis, specific proteolytic extraction/excision of carbohydrate-binding peptides, and mass spectrometric analysis of the eluted peptides is shown here to be a highly efficient tool for identifying and differentiating specific CRD structures. Pressure-assisted enzymatic hydrolysis showed an increased digestion efficiency of enzyme-resistant lectins. Despite the relatively weak interactions between peptides and carbohydrates as illustrated by the relatively high K_D values, we demonstrate here the specific differentiation between the recognition structures to carbohydrates at the submolecular level, ascertained by the binding of natural and synthetic peptides of hGal-3. The only difference between A-trisaccharide and B-trisaccharide, the N-acetylation of A-tri, is also the marker that differentiates blood group A from blood group B types. The remarkable capability of the peptide hGal-3(130–144) to differentiate between these two carbohydrate structures may render peptide epitopes a potential alternative to much larger blood group A determinant proteins, such as the *Dolichos biflorus* (anti-A1) [20]. While the single peptide hGal-3(130–144) cannot have agglutinating properties, branched polypeptide bioconjugates [26, 27] exhibiting multiple peptide ligand copies may lead to well amenable alternatives to lectins and human sera as blood-typing reagents. These results indicate the potential of pressure-assisted proteolytic excision- and extraction-mass spectrometry for identifying affinity-derived lectin epitope peptides, with the potential to be valuable therapeutic agents, or reagents for investigating cell surface carbohydrates and associated pathophysiological processes.

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