Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 840 (2006) 29–36
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Exploring the binding profiles of ConA, boronic acid and WGA by MALDI-TOF/TOF MS and magnetic particles☆

Katrin Sparbier*, Thomas Wenzel, Markus Kostrzewa

Bruker Daltonik GmbH, Permoserstrasse 15, 04318 Leipzig, Germany

Received 12 January 2006; accepted 17 June 2006
Available online 12 July 2006

Abstract

Concanavalin A, boronic acid and Wheat germ agglutinin functionalized magnetic micro-particles were developed to enrich glycosylated peptides and proteins. The bead functionalities were validated according to their specificity by analyses of model proteins. Validated beads were employed for the enrichment of glycosylated human serum proteins. Eluted glycoproteins were digested by trypsin and the resulting peptides were purified by magnetic MB-HIC C8 beads. Each fraction was analyzed by MALDI-TOF MS and single peaks were subjected to MALDI-TOF/TOF MS with the objective to identify the respective proteins by database search. Search results revealed overlapping profiles of known serum glycoproteins.

Keywords: Serum glycoproteins; MALDI-TOF/TOF MS; Magnetic particles

1. Introduction

Glycosylation is with at least 50% the most common form of post-translational modification of proteins. Degree and type of glycosylation depend on the status of cells and might be linked to certain diseases [1]. Therefore, the discovery and identification of those modified peptides and proteins gain more and more importance. MALDI-TOF mass spectrometry is an ideal technique for identifying peptides and proteins and their corresponding modifications which depends on an appropriate sample preparation. Enrichment of glycosylated peptides and proteins from different sources – the prerequisite for MALDI-TOF mass spectrometry – can be attained by chromatographic methods like lectin affinity chromatography or boronic acid capturing supported by functionalized magnetic particles.

Concanavalin A (ConA), boronic acid and Wheat germ agglutinin (WGA) functionalized magnetic particles were developed to enrich glycosylated peptides and proteins. ConA specifically binds mannosyl and glucosyl residues of polysaccharides and glycoproteins [2] containing free hydroxyl groups at positions C3, C4 and C6 [3,4] and can be applied as a general tool for capturing of *N*-glycosylated peptides and proteins with broad specificity. In contrast, boronic acid forms covalent bonds to molecules containing *cis*-diol groups [5], like mannose, galactose or glucose. Therefore, it additionally facilitates the enrichment of the more heterogeneous O-linked oligosaccharides. A further possibility for glycoprotein enrichment provides WGA. The receptor sugar for WGA is *N*-acetylglucosamine preferentially binding to dimers and trimers of this sugar. WGA can also bind oligosaccharides containing terminal *N*-acetylglucosamine, sialic acids or chitobiose which are common to many serum and membrane glycoproteins [6]. The binding motifs for the lectins and the reaction scheme for the formation of the heterocyclic boronic acid diester are shown in Fig. 1. Sepharose or agarose particles functionalized with either ConA, WGA or boronic acids have previously been applied for the successful isolation of glycosylated proteins [7–10]. The use of magnetic particles as support for the functionalization allows for easy miniaturization and automation of assays and thereby facilitating high through-put analyses.

The bead functionalities were validated according to their specificity by analyses of different model proteins, i.e. bovine serum albumin (BSA), bovine Lactoferrin, and bovine RNase B. Validated beads were employed for the enrichment of glycosylated human serum proteins. After elution the isolated

☆ This paper was presented at the Swiss Proteomics Society 2005 Congress, Zurich, Switzerland, 5–7 December 2005.

* Corresponding author. Tel.: +49 341 2431 464; fax: +49 341 9625 215.
E-mail address: KSP@BDAL.de (K. Sparbier).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.06.028

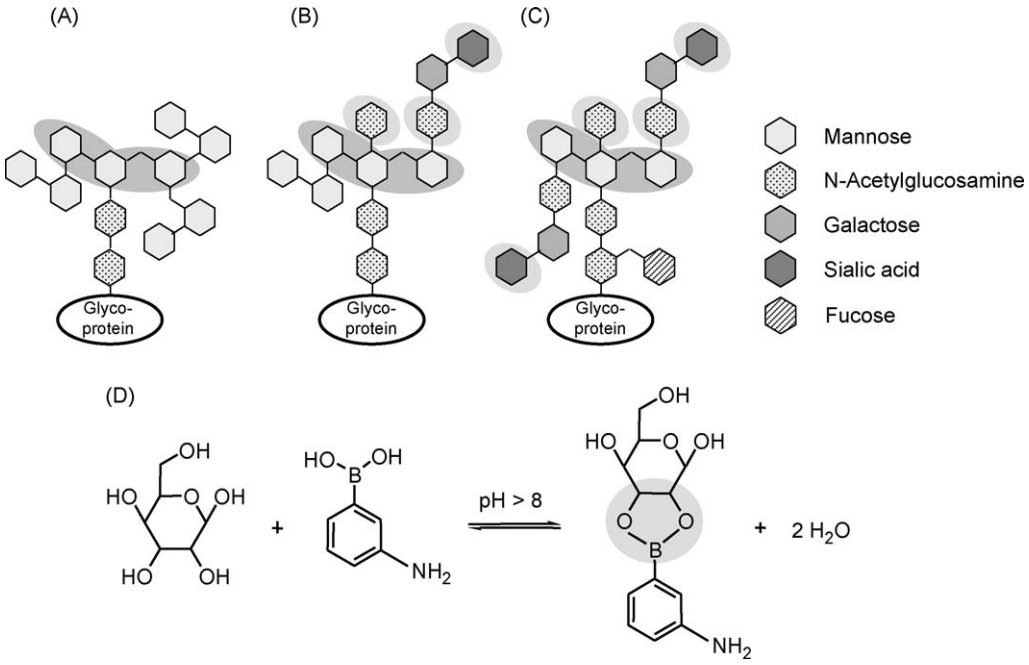

Fig. 1. Scheme of different *N*-glycan structures (A–C). (A) High-mannose type; (B) hybrid type; (C) bi-antennary complex type. The binding motifs for ConA and WGA are highlighted in dark and light grey, respectively. (D) Reaction scheme describing the formation of the pentamerous heterocyclic diester derived from mannose and 3-aminophenyl boronic acid. The heterocyclic diester is highlighted in light grey.

glycoproteins were digested by trypsin and the resulting tryptic peptides were purified using ClinProt beads MB-HIC C8. The complexity of the samples was reduced by stepwise elution from the beads. Each fraction was analyzed by MALDI-TOF MS in the reflector mode. Suitable peptide peaks were analyzed by MALDI-TOF/TOF MS with the view to identification of the corresponding proteins by database search. The database searches of fragmented peptides revealed known serum proteins containing glycosylation sites, e.g. Complement component 3, Histidine-rich glycoprotein and Amyloid P component. The different types of glyco-capturing magnetic particles represent overlapping binding profiles.

2. Experimental

2.1. Materials

Model proteins bovine serum albumin (BSA), bovine Lactoferrin, bovine RNase B) and chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany). Functionalized magnetic particles were available from Bruker Daltonik GmbH (Germany) as kits including all buffers and solutions needed for preparation. Blood samples were taken from healthy volunteers and serum was obtained after clotting for 30 min at room temperature and subsequent centrifugation.

2.2. Binding and elution of captured proteins

Binding of proteins and serum samples onto the magnetic particles was performed according to the manufacturer's recommendations. Briefly, for ConA and WGA beads model proteins (1 μg each) or serum (20 μl) were incubated for 1 h at RT under gentle agitation and neutral binding conditions. The beads were separated from the supernatant using a magnetic separation device (Bruker Daltonik GmbH, Germany). After washing of the beads the bound glycoproteins/-peptides were eluted under acidic conditions. Binding of model proteins (1 μg each) or serum (20 μl) onto boronic acid functionalized beads was performed under slightly alkaline (pH 8.5) conditions. After incubation for 1 h at RT under gentle shaking the beads were washed and the bound protein species were eluted under acidic conditions.

2.3. Competition experiments

For competition experiments, binding of glycoproteins was performed in the presence of 500 mM α-methylmannoside for ConA beads, 500 mM *N*-acetylglucosamine for WGA beads and 1.5 M sorbitol for boronic acid beads.

2.4. Tryptic digestion of elution products

Eluates from the magnetic particles were dried in a Speed-Vac. The resulting pellets were dissolved in 50 μl 100 mM NH_4HCO_3 pH 8.2. Each vial was supplemented with 0.1 μg of trypsin and was incubated overnight at 37 °C. Digests were analyzed by MALDI-TOF MS in the reflector mode before further purification.

2.5. Purification of tryptic peptides

Tryptic peptides were purified using MB-HIC C8 beads (Bruker Daltonik GmbH, Germany). For purification, 10 μl beads and 5 μl 10% TFA were added to the digests. After

binding subsequent washing steps were performed according to the manufactuer's recommendations. Complexity of the purified peptides was reduced by stepwise elution using 5 μl of 10%, 20%, 30%, 40%, and 50% of acetonitrile as elution solution, respectively.

2.6. Mass spectrometry

2.6.1. Mass spectrometry of proteins

For MS analysis of undigested proteins the eluted samples were prepared onto AnchorChip^{TM} 600 targets (Bruker

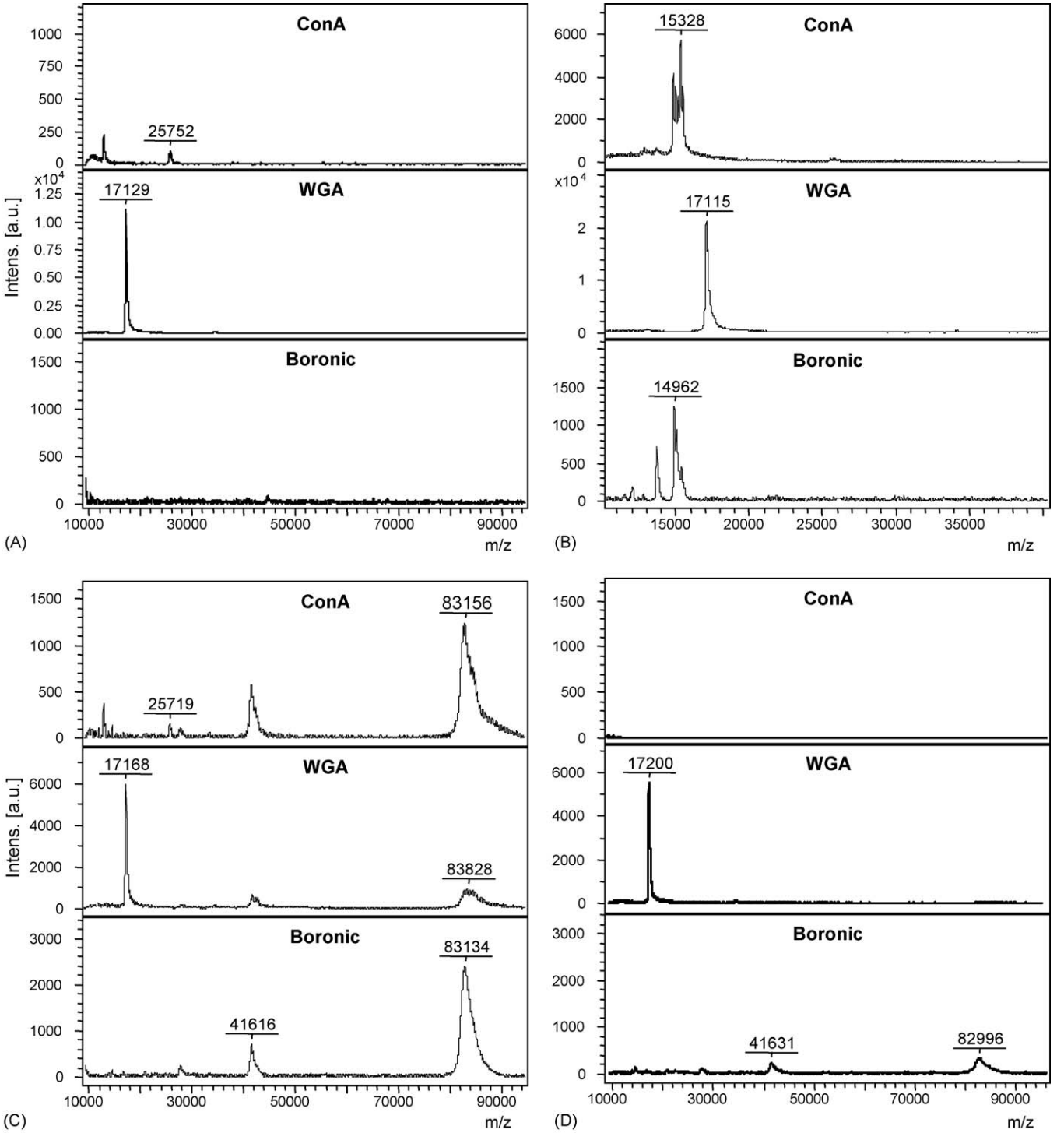

Fig. 2. (A) Binding of BSA (66 kDa), (B) RNase B (14.9–15.3 kDa) and (C) Lactoferrin (83 kDa) by ConA, WGA and boronic acid beads, respectively. Peaks at 17 and 25 kDa represent the lectins WGA and ConA. (D) Competition assay: Binding of Lactoferrin in the presence of 500 mM α-methylmannoside, 500 mM *N*-acetylglucosamine and 1.5 M sorbitol by ConA, WGA and boronic acid beads, respectively. The estimated relative amount of bound protein ranged from 90 to 100% for Lactoferrin and about 70–80% for RNase B for all three bead types.

Daltonik, Germany) using HCCA (α-cyano-*p*-hydroxycinnamic acid; Bruker Daltonik GmbH, Germany) and 2,5-dihydroxyacetophenone (2,5-DHAP) (Bruker Daltonik, Germany) as matrices according to the manufacturer's recommendations. Peptide Standard I (Bruker Daltonik GmbH, Germany; containing Bradykinin, Angiotensin I, Angiotensin II, Substance P, Bombesin, ACTH (1–17), ACTH (18–39) and Somatostatin), Protein Standard I (Bruker Daltonik GmbH; containing Insulin, Ubiquitin, Cytochrom C and Myoglobin) and Protein Standard II (Bruker Daltonik GmbH, Germany; containing Trypsinogen,

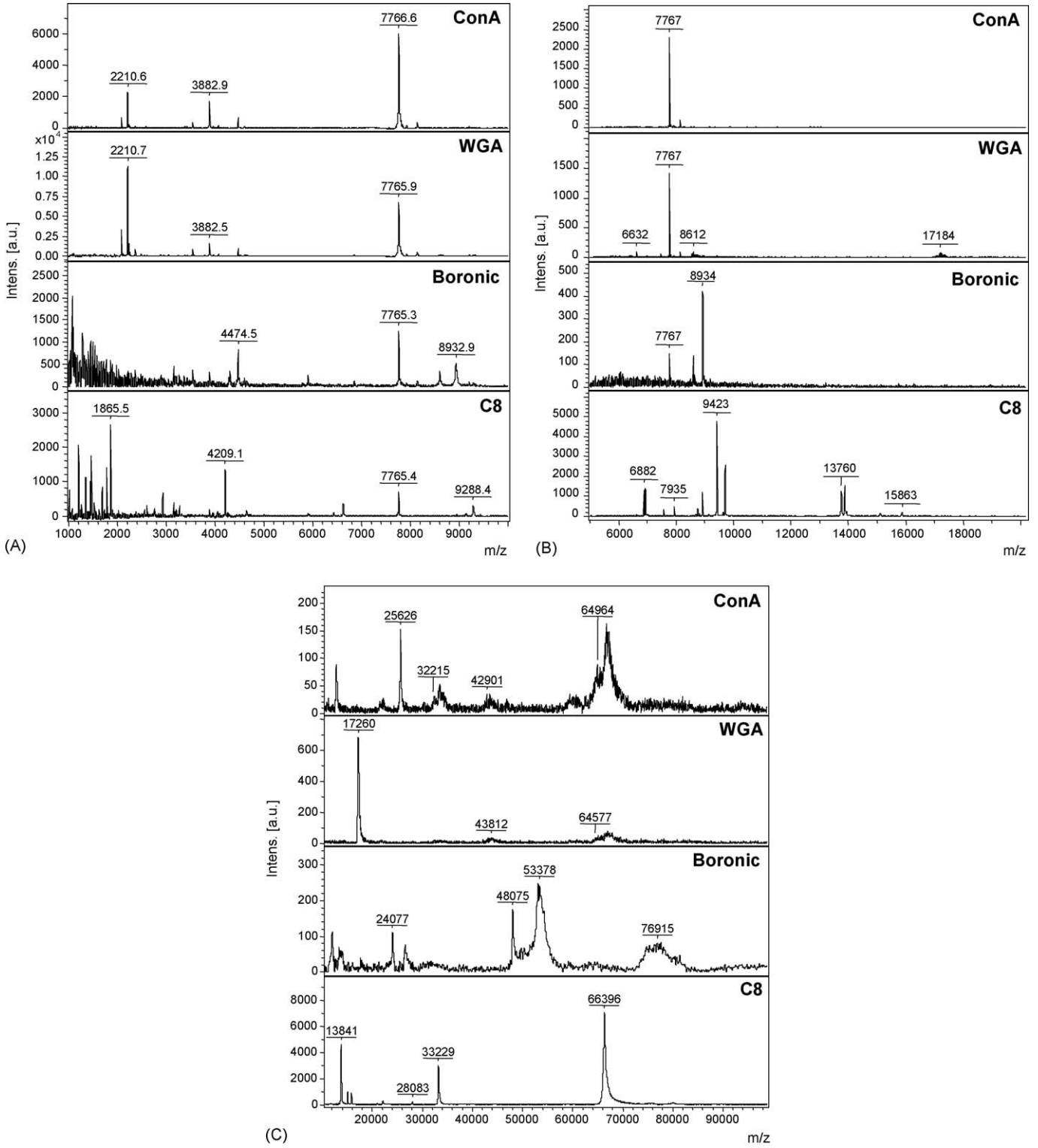

Fig. 3. Binding of serum peptides and proteins by ConA, WGA, boronic acid and hydrophobic C8 beads. Mass spectra were acquired in an autoflex II TOF/TOF in the mass range from 1 to 10 kDa using HCCA (A), from 5 to 20 kDa (B) and from 10 to 100 kDa (C) employing 2,5-DHAP as matrix.

Protein A and BSA) were used for calibration of the respective mass range. MS measurements were performed in an autoflex II TOF/TOF (Bruker Daltonik GmbH, Germany). Spectra were acquired in the linear mode in the mass ranges from 1 to 10, 5 to 20 and 10 to 100 kDa. Five hundred single spectra (10 × 50 shots) were summarized at 50 Hz for each sample applying the following instrument settings (mass range 10–100 kDa)—ion source 1: 20.0 kV, ion source 2: 17.7 kV, lens: 8.7 kV, pulsed ion extraction: 500 ns, the detector gating was set to 8000 Da.

2.6.2. Mass spectrometry of tryptic peptides

Peptides were prepared with HCCA on AnchorChip™ 600 targets according to the manufacturer's recommendations. Peptide Standard I was employed for calibration. MS spectra were acquired in an autoflex II TOF/TOF in the reflector mode by summarizing 90 single spectra (3 × 30) with a 50 Hz laser in the mass range from 900 to 3500 Da applying the following instrument settings—ion source 1: 19.00 kV, ion source 2: 16.75 kV, lens: 8.90 kV, reflector1: 20 kV, reflector 2: 9.50 kV, reflector detector: 1739 V, suppression up to 600 Da by deflection.

MS/MS spectra of tryptic peptides were acquired in an autoflex II TOF/TOF in the MS/MS mode using the following instrument settings—ion Source 1: 6.00 kV, ion Source 2: 5.30 kV, lens: 3.15 kV, reflector 1: 23.50 kV, reflector 2: 9.90 kV, lift 1: 18.00 kV, lift 2: 4.00 kV, reflector detector: 1732 V.

2.7. Database search

Fragment ion spectra were submitted to MASCOT (http://www.matrixscience.com/) for database search and identification of corresponding proteins employing the following search parameter settings—Database: SwissProt 49.5 (279981 sequences; 129283946 residues) restricted to Homo sapiens (human) (60785 sequences), type of search: MS/MS Ion Search, enzyme: Trypsin, variable modifications: oxidation (M), mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ±0.2 Da, fragment mass tolerance: ± 0.8 Da, max missed cleavages: 1, instrument type: MALDI-TOF-TOF. Only those protein hits were considered for evaluation which were based on at least one peptide MS/MS exceeding the MASCOT identity threshold ($p = 0.05$, ID threshold= 36).

3. Results and discussion

3.1. Demonstration of binding specificity

The binding specificity of functionalized beads was demonstrated for several model proteins (Fig. 2A–C). BSA (66 kDA) which is a non-glycosylated protein was used as a negative control and was not bound by any of the beads (Fig. 2A). The peaks at 25 kDa in the ConA spectrum and at 17 kDa in the WGA spectrum represented the monomers of ConA and WGA, respectively. Fig. 2B shows the binding of RNase B (14.9–15.3 kDa) by the different bead types. According to the high mannose *N*-glycan structure of the single glycosylation site of this protein RNase B does not present the binding motif for WGA [11] and was not captured. The *N*-glycan structure of RNase B comprises different numbers of mannosyl residues resulting in several individual peaks in the MS spectrum. Capturing of RNase B with ConA revealed a preferential binding of protein molecules with high numbers of mannosyl residues resulting in a main peak at 15.3 kDa. In contrast, boronic acids bound RNase B according to the natural distribution represented by a main peak at 14.9 kDa. Further, some unspecific contamination at about 13 kDa was observed in the case of boronic acids. Bovine Lactoferrin (83 kDa) is a glycoprotein with five and four *N*-glycosylation sites for Lactoferrin isoform-a and -b comprising two high mannose *N*-glycan structures, two hybrid structures and one complex type structure in the b-isoform. According to the different *N*-glycan structures of the Lactoferrin glycosylation sites [12], this protein was bound by all three-bead types (Fig. 2C). The binding specificities of the three different bead types were further validated by competition experiments. Binding of Lactoferrin was performed in the presence of competing agents: 500 mM α-methylmannoside, 500 mM *N*-acetylglucosamine and 1.5 M sorbitol for ConA beads, WGA beads and boronic acid beads, respectively. For ConA and WGA beads the binding of Lactoferrin was completely suppressed in the presence of the corresponding competitor. In the case of boronic acid functionalized beads the binding was only reduced but not completely prevented. This might be due to the high number of available binding sites on the boronic acids functionalized beads. The results are depictured in Fig. 2D.

3.2. Isolation of glycosylated serum proteins and peptides

Glycosylated peptides and proteins from human serum were isolated using the three different bead types, ConA beads, WGA beads and boronic acid functionalized beads, respectively. For comparison, the same serum was purified by hydrophobic beads (MB-HIC C8). The eluates were directly analyzed by MALDI-TOF MS. Spectra were acquired for different mass

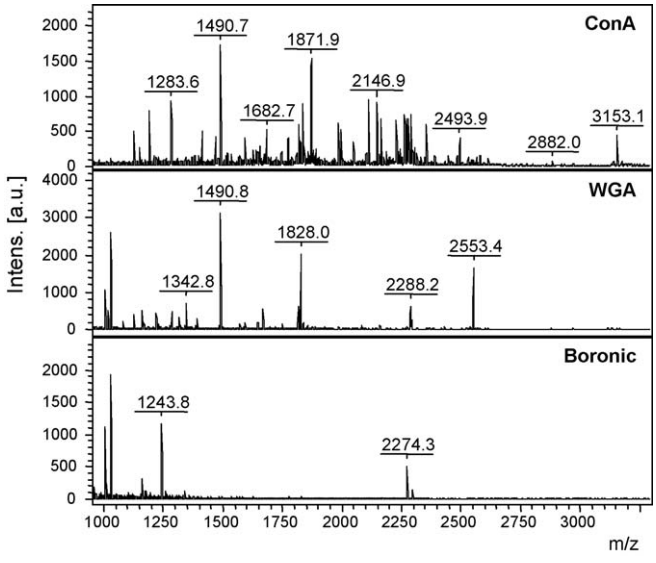

Fig. 4. MALDI-TOF MS reflector spectra of purified tryptic peptides after enrichment by ConA, WGA and boronic acid beads. The mass spectra of the 30% acetonitrile elution step are displayed.

ranges in the linear mode using HCCA for the range from 1 to 10 kDa and 2,5-DHAP for the ranges 5–20 kDa and 10–100 kDa as matrix, respectively (Fig. 3A–C). The resulting spectra of the eluates from ConA, WGA and boronic acid functionalized beads revealed the isolation of different as well as identical peptide and protein species. In the case of the lectin beads the masses at 25 and 17 kDa represented the lectins ConA and WGA, respectively, which were also partly released from the beads during the elution procedure. In the mass range from 1 to 20 kDa high resolution spectra could be acquired. The number

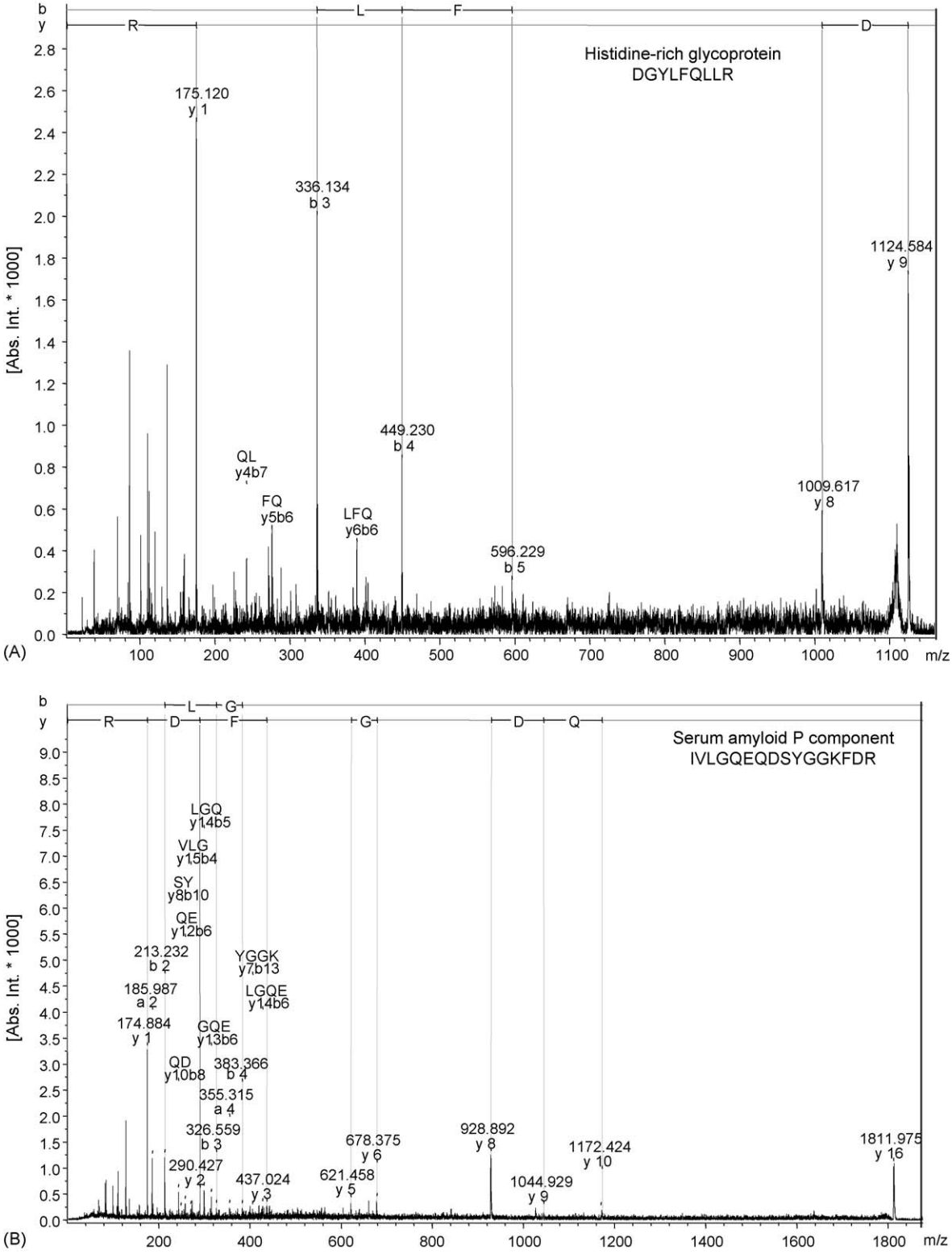

Fig. 5. Examples of two MS/MS spectra acquired on an autoflex II TOF/TOF. (A) MS/MS spectrum of the peptide peak $m/z = 1124.5$ Da representing the peptide aa 44–52 of Histidine rich glycoprotein. (B) MS/MS spectrum of the peptide peak $m/z = 1811.9$ Da representing the peptide aa 150–165 of Serum Amyloid P component.

of distinct peaks detected in this range was drastically reduced compared to the simple hydrophobic beads. This observation was in accordance to the specificity of the glyco-structure binding beads. In the mass range from 10–100 kDa only few peaks were detectable although a high number of glycosylated serum proteins are described for this mass range [13]. This might be due to the high complexity of the samples leading to suppression of single peaks, the weak ionisation efficiency of proteins and glycoproteins of higher masses and the tendency of high mass proteins for fragmentation. For ConA and WGA beads distinct peaks at about 28.000, 43.000 and 66.000 Da were observed revealing some unspecific binding of Human Serum Albumin (66 kDa) and of Apolipoprotein A I (28 kDa), respectively. Further, some hints of additional peaks were visible. Compared to an unspecific purification employing hydrophobic beads (e.g. MB-HIC C8) the unspecific contaminations were relatively marginal (Fig. 3C). Apolipoprotein A I and HSA were the main components isolated by C8 beads. Employing boronic acids beads a number of additional, distinct peaks in the mass range from 20 to 100 kDa was observed. Apart from the known high abundant proteins no further species could be directly identified by this approach. For characterization and identification of enriched peptides and proteins advanced processing of the samples was required.

3.3. Characterisation and identification of isolated serum glycoproteins

Human serum was fractionated by the different glycostructure binding magnetic beads and the eluates were directly digested by trypsin. The resulting peptides were further purified by MB-HIC C8 beads in combination with stepwise elution (10%, 20%, 30%, 40%, 50% ACN) to reduce the sample complexity. MALDI-TOF MS reflector spectra were acquired for each fraction. Exemplarily, the spectra of the fractions of ConA, WGA and boronic acid beads eluted with 30% ACN are displayed in Fig. 4. All peaks above an intensity threshold of 500 relative intensity units and separated from neighbouring peaks by at least 10 Da were subjected to MS/MS analysis. In total 55 peaks from the ConA fractions, 43 peaks from the WGA fractions and 37 peaks from the boronic acid fractions were analysed by MS/MS. Examples for the MS/MS spectra of a tryptic peptide of Histidine-rich glycoprotein (1124.582 Da) and of a tryptic peptide of Serum Amyloid P component (1811.971 Da) are given in Fig. 5. The resulting fragment spectra were submitted to MASCOT for database search with the objective to identify the corresspponding proteins. For the ConA fraction 45 MS/MS spectra, for the WGA fraction 21 spectra and for the boronic acid fraction 17 spectra led to significant hits revealing 12, 10 and 10 different maternal proteins, respectively. The binding profiles of the different beads comprised different and also identical proteins. Alpha-2-macroglobulin, Ceruloplasmin and Histidine-rich glycoprotein were bound by ConA and WGA. Kininogen was found in the WGA and the boronic acid fraction and Complement C1q was detected by ConA and boronic acid. The individual but overlapping binding profiles which had been determined by a combination of specific glyco-capturing beads, tryptic digest and bead based reversed phase purification are depictured in Fig. 6. The applied simple strategy allowed for the identification of 18 glycosylated serum proteins comprising five serum glycoproteins contributing to the fraction making up 10% of the serum proteins and six glycoproteins contributing to the fraction making up 1% of serum proteins. The pie chart in Fig. 7 represents the relative contribution of the identified proteins within the serum [13]. Two cell surface receptor proteins were bound: Cadherin-10 by ConA and Cytokine receptor common β chain by boronic acid. Since shedding of membrane proteins is a common event it is very likely that the two glycosylated proteins were captured from serum. Together, four unspecifically bound proteins each represented by only single tryptic peptides were detected. In the ConA captured fraction two non-glycosylated proteins (human serum albumin (HSA), apolipoprotein AI)

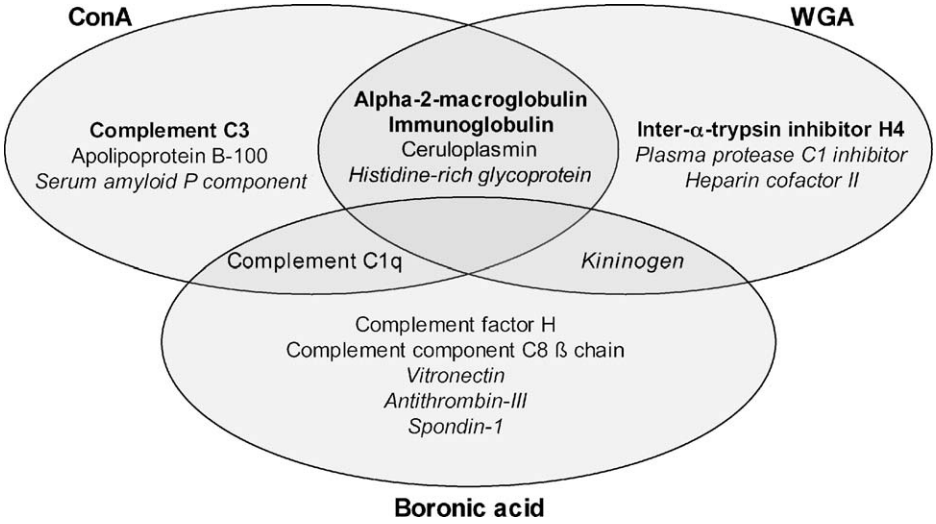

Fig. 6. Display of the overlapping, but individual binding profiles of MB-LAC ConA, MB-LAC WGA and MB-CovAC boronic beads. High abundant proteins are written in bold letters. Proteins belonging to the fraction making up 1% of serum proteins are written in italics and proteins of the fraction making up 10% of the serum proteins are displayed in normal letters.

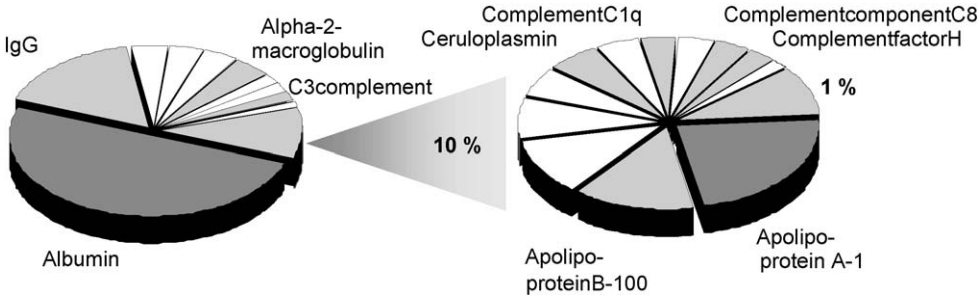

Fig. 7. Pie chart representing the relative contribution of proteins within the serum [13]. Identified glycoprotein species were highlighted in light grey and labeled. Identified but non-glycosylated proteins were highlighted in dark grey and labeled.

were found. Additionally, one peptide fragment of ConA, which was partly eluted from the beads, was detected. All three proteins could also be detected by the direct analysis and were represented by the peaks at 66 kDa, 28 kDa and 25 kDa, respectively (Fig. 3). Furthermore, HSA and Apolipoprotein AI were also unspecifically bound by WGA. The non-glycosylated intracellular protein Gelosin and the non-glycosylated Plasma Glutathion Peroxidase were captured by boronic acids beads.

4. Conclusions

The binding specificity of the differently functionalized magnetic particles was demonstrated by competition experiments. Incubation of beads and glycoproteins in the presence of the corresponding competitor prevented binding of glycoproteins. The detailed analysis of ConA, WGA and boronic acid enriched serum proteins by tryptic digestion and MS/MS analyses revealed the specific binding of nine glycosylated proteins by ConA, eight glycosylated proteins by WGA and eight glycoproteins by boronic acid. Only four non-glycosylated peptides were identified. The employed strategy allowed for the detection of eight glycoproteins belonging to the low abundant fraction of serum proteins. Each bead type presented its own individual binding profile overlapping with the profiles of the two others. The employment of functionalized magnetic bead based techniques in conjunction with mass spectrometry combines short processing times and automatic workflows with high-resolution analyses.

Acknowledgements

This work has been supported by the Sächsische Aufbaubank grant 7495/1185. We thank Irina Keßler for technical assistance.

References

[1] G. Durand, N. Seta, Clin. Chem. 46 (2000) 795.
[2] A. Kamra, M.N. Gupta, Anal. Biochem. 164 (1987) 405.
[3] I. Yahara, G.M. Edelman, PNAS 69 (1972) 608.
[4] I.J. Goldstein, C.E. Hollerman, E.E. Smith, Biochemistry 41 (1965) 876.
[5] J.D. Rawn, G.E. Lienhard, Biochemistry 13 (1974) 3124.
[6] Y. Nagata, M.M. Burger, J. Biol. Chem. 240 (1974) 3116.
[7] R. Lotan, G. Beattie, W. Hubbell, G.L. Nicolson, Biochemistry 16 (1977) 1787.
[8] G.A. Bloomfield, M.R. Faith, J.G. Pierce, Biochim. Biophys. Acta 533 (1978) 371.
[9] K. Yamamoto, I. Tsuji, I. Matsumoto, T. Osawa, Biochemistry 20 (1981) 5894.
[10] C. Gerard, Methods Enzymol. 182 (1990) 529.
[11] T. Liu, J.D. Li, R. Zeng, X.X. Shao, K.Y. Wang, Q.C. Xia, Anal. Chem. 73 (2001) 5875.
[12] Z. Wei, T. Nishimura, S. Yoshida, J. Dairy Sci. 84 (2001) 2584.
[13] L.A. Liotta, E. Petricoin, Clin. Prot. 1 (2004) 101.