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Application of a pH responsive multimodal hydrophobic interaction chromatography medium for the analysis of glycosylated proteins

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ABSTRACT

Protein glycosylation has significant effects on the structure and function of proteins. The efficient separation and enrichment of glycoproteins from complex biological samples is one key aspect and represents a major bottleneck of glycoproteome research. In this paper, we have explored pH multimodal hydrophobic interaction chromatography to separate glycosylated from non-glycosylated forms of proteins. Three different proteins, ribonuclease, invertase and IgG, have been examined and different glycoforms have been identified. The media itself shows strong responsiveness to small variations in pH, which makes it possible to fine-tune the chromatographic conditions according to the properties of the protein isolated. Optimal glycoprotein separation has been obtained at pH 4. The pH responsive multimodal HIC medium in contrast to conventional HIC media is able to resolve contaminating DNA.

1. Introduction

The majority of eukaryotic proteins are glycoproteins [1,2]. The glycosylation has large impacts on protein properties and the carbohydrate moieties frequently play key roles in mediating and modulating cell signalling and receptor binding [3,4]. The degree and form of glycosylation can vary at a given glycosylation site, which may lead to a heterogeneity in protein glycoforms. For instance, recombinant tissue plasminogen activator (tPA) has been estimated to contain up to 11,500 different glycoforms [5]. Different glycoforms often have different activities and occasionally glycosylation of a protein can result in a protein with an entirely different function compared to the native protein. As a consequence of this, one variant of a glycoprotein might be pharmaceutically active while others may appear immunogenic or even toxic. The identification and analysis of different protein glycoforms are therefore essential in drug development and the different factors influencing glycosylation have been studied in detail [6,7]. In addition, the possibilities to genetically design glycosylation patterns (glycoengineering) are getting increasingly important to modify the properties of a protein [8–10].

Traditionally, proteins are isolated and purified in a series of steps based on various chromatographic techniques such as affinity, hydrophobic interaction (HIC) or ion-exchange chromatography (IEC). However, the development of chromatographic systems for glycoprotein separations has been slow and only little is known on how glycosylation affects the chromatographic behaviour of proteins. Boronate affinity [11], capillary electrophoresis [12], and various HPLC methods such as lectin chromatography [13,14], ion-exchange chromatography [15,16] and reversed phase chromatography [17] have all been applied for separating glycoproteins, but these techniques often prove insensitive to the small structural changes that glycosylation modifications present. A pH responsive multimodal HIC media has previously been described and used to detect minor structural modifications of proteins including single amino acid substitutions [18–20]. The use of multimodal separation media where the binding ligands are designed to allow for multiple or specific interactions, thus often increases selectivity and specificity of a chromatographic separation. In this study we have therefore examined the retention behaviour of proteins which vary in their degree of glycosylation using multimodal HIC media. Compared to traditional media the multimodal media can effectively distinguish between various protein subpopulations.

The pH responsive HIC (pH HIC) medium used in the present studies was obtained from the Custom Designed Media group at GE Healthcare (Uppsala, Sweden). It is a polymeric coating applied or

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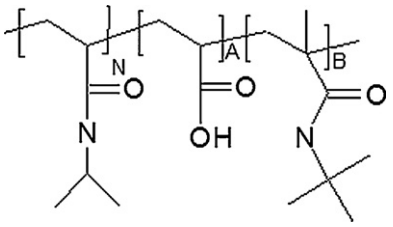

Fig. 1. General structure of the pH HIC ligand used.

generated at the surface of an existing base matrix—in the present studies Sepharose™ High Performance (HP). The general synthesis protocol of the media has previously been published [21]. It is a copolymer of three different functional groups, N-isopropyl acryl amide (NIPAAm), acrylic acid (AA) and butyl acrylamide (BAA) (Fig. 1). The latter can be in linear or tert-butyl form (t-BAA). NIPAAm provides for "hydrophobic effect" driven self-association at temperatures above a critical temperature (typically in range 30–40 °C depending on solution conductivity and other operating conditions). The biomedical uses and bioengineering applications for such polymers have been discussed [22,23]. The employment of AA groups results in polymers, which can alter their hydrophobicities over a pH range (4–8) suitable for especially protein chromatography. This shifts the self-association responsiveness of the polymer from being solely temperature dependent to pH plus temperature. In addition, BAA, or the more chemically stable t-BAA used in the present media, allows for a tuning of the media performance. We and others [19,20,24–26] have previously described protein separations on this type of media under various combinations of pH and temperature.

In the present study we have focused on applying a pH responsive HIC medium to the analysis of glycosylated proteins. We have examined three different glycoproteins, ribonuclease (RNase), invertase and a monoclonal antibody using conventional phenyl and butyl media in comparison with a pH multimodal HIC matrix. Special emphasis has been placed on the identification of optimal chromatographic conditions, such as pH and ionic strengths, to optimise separations. We have also attempted to monitor common nucleic acids contaminants during the separations and the ability of the chromatographic materials to remove these.

2. Materials and methods

2.1. Sample preparation and chemicals

The recombinant human monoclonal IgG1 feedstocks X and Y consisted of clarified cell culture harvest expressed in CHO or NS0 cells using conventional manufacturing process steps at BioInvent International AB (Lund, Sweden). The clarified feedstocks were purified by protein A chromatography and eluted at acidic pH. The multimodal pH-HIC chromatography resins were obtained from GE Healthcare (Uppsala, Sweden) and have previously been described [17]. Yeast invertase and RNase A and B were purchased from Sigma–Aldrich (St. Louis, MO, USA). Quant-iT PicoGreen dsDNA kit was from Invitrogen (Carlsbad, CA, USA). All chemical reagents were of analytical grade and obtained from Sigma–Aldrich or Merck (Darmstadt, Germany) unless otherwise specified.

2.2. Enzymatic deglycosylation

2 mg of RNase B or yeast invertase was dissolved in 100 μL 50 mM citrate buffer pH 5.5 and digested with 0.02 units Endoglycosidase H (Roche Diagnostics, Mannheim, Germany). The reaction was carried out overnight at 37 °C in the presence of 0.1% sodium azide. Similarly, 0.4 mg of the monoclonal antibody Y was digested in 200 μL reaction buffer G7 by 2000 NEB units PNGase F (New England Biolabs, Ipswich, MA, USA). The reaction was carried out overnight unless otherwise specified at 37 °C in the presence of 0.1% sodium azide. The deglycosylation of the proteins was confirmed by SDS-PAGE on precast 4–20% Mini-Protean TGX gels (BioRad, Richmond, CA, USA).

2.3. Chromatography conditions

The multimodal pH responsive HIC media was packed to a volume of 1 mL in a HR 5/5 column. Butyl- and Phenyl Sepharose HP was purchased prepacked in 1 mL HiTrap columns. For the pH screening of RNase binding the column was packed to a volume of 700 μL. All experiments were carried out on an ÄKTA explorer 10 system (GE Healthcare, Uppsala, Sweden).

2.4. Isocratic elution conditions

Isocratic elution of RNase A and B was performed at a conductivity of 200 mS/cm in 50 mM citrate buffer pH 4.0 supplemented by 1.65 M ammonium sulfate. The column was equilibrated for 10 column volumes (CV) followed by injection of 0.2 mg protein at a constant flow rate of 30 cm/h.

2.5. Gradient elution conditions

The columns were equilibrated for 10 CV followed by an injection of 0.1 mg protein in 50 μL and 10 CV washing. Bound proteins were eluted with a 20 CV linear gradient. All experiments were carried out with a buffer system composed of 50 mM citrate buffer pH 4.0 with 2.25 or 1 M ammonium sulfate except for the binding and elution of antibodies from Butyl Sepharose where a 50 mM Tris–HCl buffer pH 9.0 was employed.

2.6. MALDI

Prior to the MALDI analysis, the PNGaseF digested IgG was purified on porous graphite carbon (PGC) columns (Dalco Chromtech, Sweden). The columns were equilibrated with 0.1% TFA, 5% MeCN. After sample application the columns were washed with MilliQ water and 0.1% TFA, 5% MeCN. The glycans were then eluted and recovered in 0.1% TFA, 50% MeCN. The samples were subsequently dried in vacuum at 35 °C.

The glycans were analyzed using a 2,5-dihydroxybenzoic acid (DHB) matrix (Bruker Daltonik GmbH, Germany) in 0.1% TFA, 5% MeCN. The dry eluate was dissolved in 10 μL 10 μM NaCl, 0.1% TFA. 2 μL sample was applied on the target plate (MSP 96 target ground steel (Bruker Daltonik GmbH, Germany)) followed by 0.5 μL DHB. Mass spectra were acquired using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The data was collected in positive ion reflectron mode with a delayed extraction of 200 ns. Each mass spectra was generated from 850 shots and the mass range was 600–2800 Da. Ion source voltage 1 was 19.0 kV and ion source voltage 2 was 16.4 kV. The spectra were analyzed using Flexanalysis 3.0 software, and the masses were annotated to glycans with Glyco-Peakfinder.

3. Results and discussion

3.1. pH response and glycosylation effects

Bovine pancreatic ribonuclease B (RNase B) is an N-linked glycoprotein composed of 124 amino acid residues with an isoelectric point of approximately 9.6. The only existing glycosylation site is

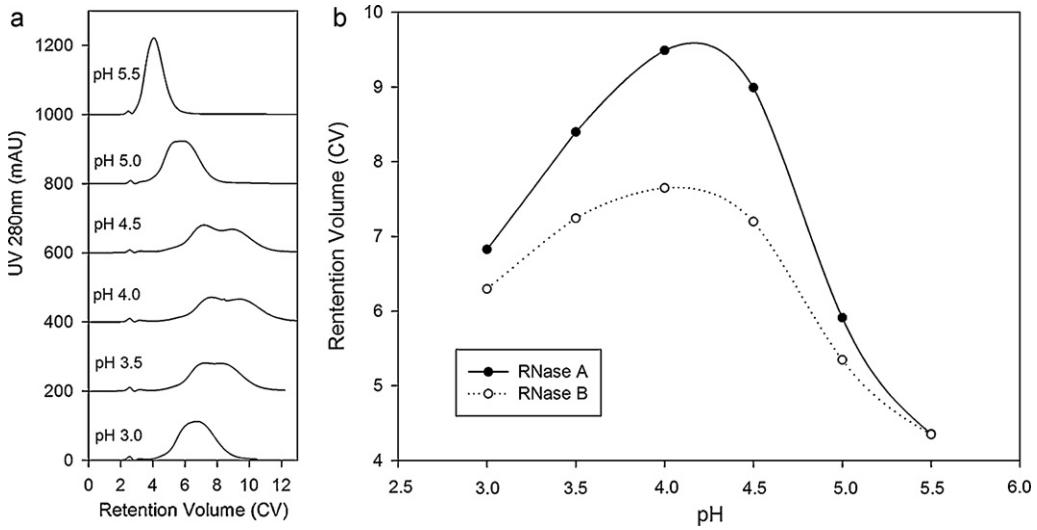

Fig. 2. (a) Chromatograms of the isocratic elution of an RNase A and B mixture at different pHs. Flow rate 0.1 mL/min, 1.65 M ammonium sulfate isocratic gradient in a 50 mM citrate buffer. (b) Retention volumes of the RNase A and B mixture at different pH values.

well documented and is located on Asn^{34} with an oligosaccharide moiety of the common high-mannose-type [27,28]. Five different glycoforms exist and each of them is present in a wide variety of isoforms [29]. The glycosylation results in an increase in molecular mass from 13,683 to 14,899–15,547 Da depending on the number of mannose units. The carbohydrate fraction is thus equivalent to 8–12% of the protein mass. RNase B is also available in a non-glycosylated variant, RNase A, which has an enhanced catalytic activity but reduced proteolytic stability.

The multimodal HIC media is highly sensitive to pH changes and to examine the selectivity of the material, a mixture of RNase A and B was applied to a 0.7 mL column of the media. The peak eluting first in the chromatogram was RNase B as it is glycosylated and therefore more hydrophilic (Fig. 2). In the pH range tested, 3.0–5.5, maximal separation was obtained at pH 4.0, which is close to the p*K*a of the HIC ligand. At pH 4.0 the peaks were separated by 1.80 CV as compared to pH 5.0 where the difference between them was 0.56 CV. Resolution decreased rapidly when the pH value was either increased or decreased and already at pH 5.5 and pH 3.0 the two peaks could not be discerned. This clearly indicates that it is possible to modify and control the properties of the separation media to fit the properties of the proteins which need to be purified. This is due to conformational changes of the polymer surface coating on the matrix which can self-associate or disassociate when changing pH. At lower pH the carboxyl group (group A, Fig. 1) can associate with the matrix-exposing group B resulting in a more hydrophobic character [19,30]. Separation processes on conventional HIC media are only mildly affected by pH, and are due to changes in the charged state of the proteins, not the media itself. Since the HIC ligand is known to have a p*K*a around 4 and RNase has a much higher p*I*, we believe that this experiment specifically demonstrates the pH responsiveness of the media.

In order to further examine how glycosylation affects retention, RNase B was enzymatically deglycosylated by Endoglycosidase H

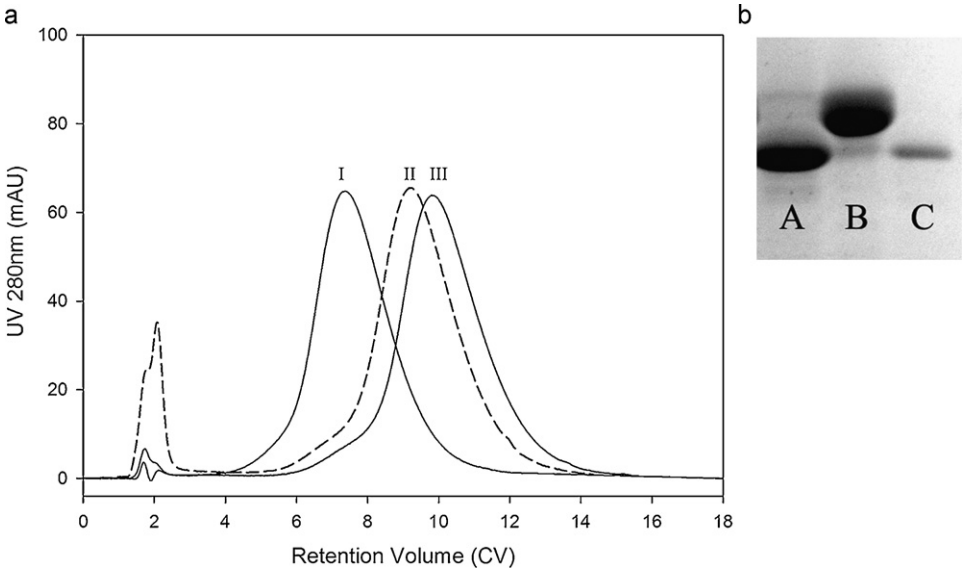

Fig. 3. (a) Comparison of RNase with different carbohydrate contents. Flow rate 0.1 mL/min, 1.65 M ammonium sulfate isocratic gradient in a 50 mM citrate buffer (I) RNase B (II) Endoglycosidase H digest of RNase B, one GlcNAc residue remains (III) RNase A. (b) SDS-PAGE digestion control. (A) Endoglycosidase H digest of RNase B; (B) RNase B; (C) RNase A. The weak band seen below (B) is an RNase A impurity present in the commercial product.

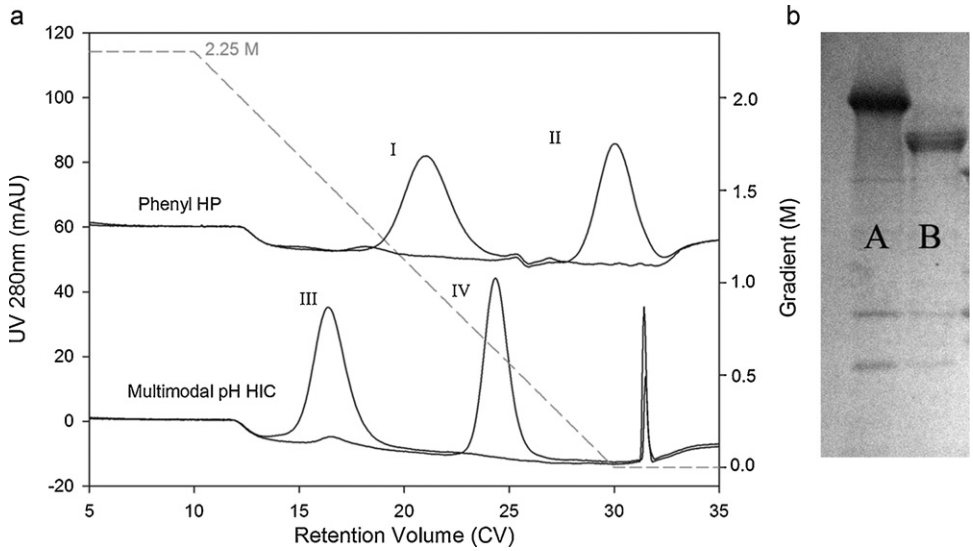

Fig. 4. (a) Comparison of the multimodal pH HIC with Phenyl HP. Flow rate 150 cm/h with a 20 CV linear gradient from 2.25 to 0 M ammonium sulfate. (I) and (III) are native invertase; (II) and (IV) are Endo H digests of invertase. (b) SDS-PAGE digestion control. (A) Native invertase and (B) Endo H digest of invertase.

(Fig. 3b). This resulted in a protein almost identical to RNase A, but with the difference that one single N-acetylglucosamine (GlcNAc) residue is still attached to Asn[34]. Fig. 3a shows that the retention shifts 1.85 CV when the carbohydrate is removed, this corresponds to 75% of the retention difference between RNase A and B, which is 2.46 CV. The difference in retention between RNase A and the deglycosylated RNase B is due to the remaining GlcNAc residue.

3.2. Comparison between multimodal and Phenyl HP media

The use of conventional HIC for glycoprotein identification is limited and we therefore compared the chromatographic behaviour between traditionally used butyl and phenyl HIC and the multimodal media. In the case of the two RNase glycoforms no separation could be achieved on butyl or phenyl matrices under any conditions tested. A single glycosylation site was simply not sufficient for these materials to allow separation. Instead, the highly glycosylated yeast invertase was used to compare the chromatographic performances of the different HIC media. In its excreted form invertase is a 270 kDa glycoprotein where the mass consists

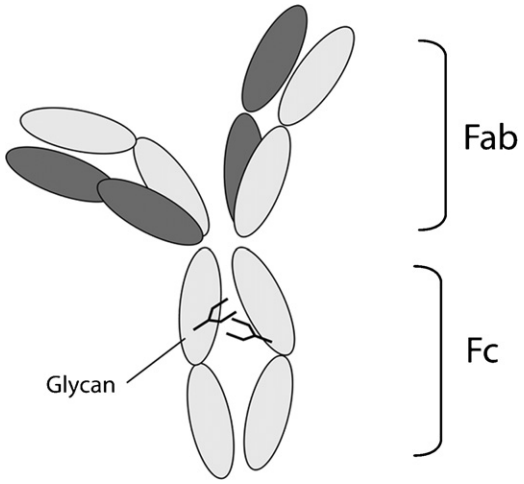

Fig. 5. Schematic drawing of an IgG antibody carrying carbohydrates on the Cγ2 domains.

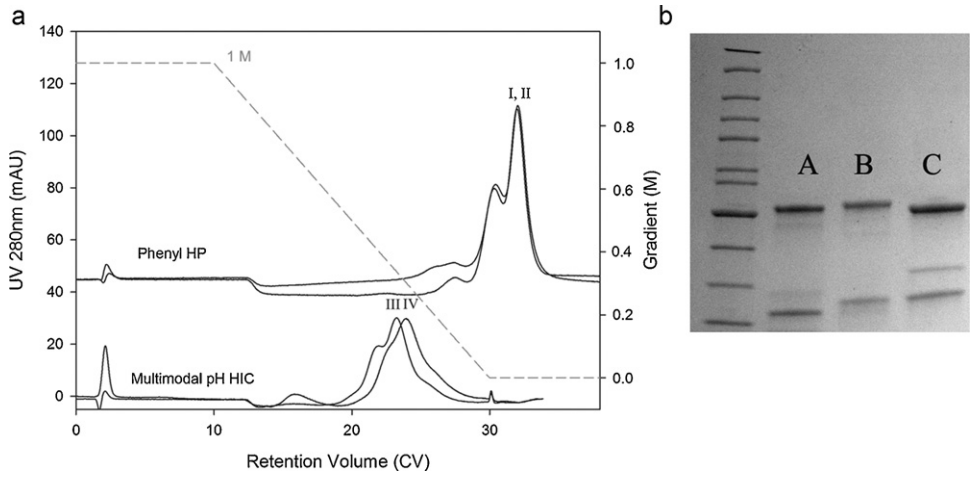

Fig. 6. (a) Separation of human mAbs and its digest on phenyl and multimodal HIC media. Flow rate 150 cm/h with a 20 CV linear gradient from 1.0 to 0 M ammonium sulfate. (I) and (III) are mAb Y; (II) and (IV) are mAb Y′. (b) SDS-PAGE digestion control. (A) mAb X; (B) mAb Y; (C) mAb Y′. The additional third band in (C) corresponds to the molecular weight of PNGase F (36 kDa).

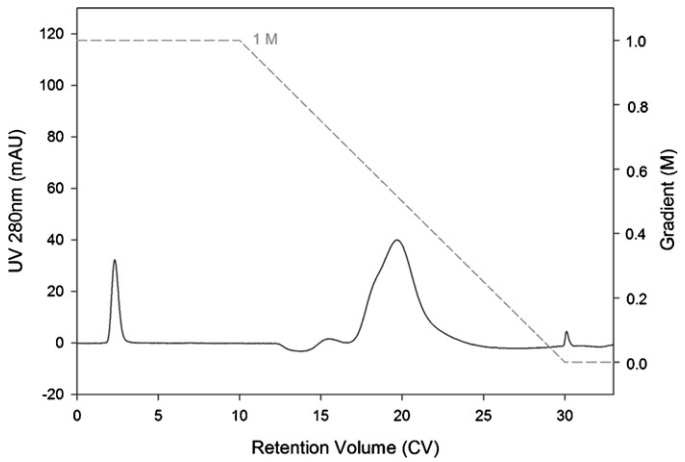

Fig. 7. Chromatogram of the natively unglycosylated mAb X. Flow rate 150 cm/h with a 20 CV linear gradient from 1.0 to 0 M ammonium sulfate on multimodal HIC media.

of 50% oligosaccharides [31]. Enzymatically removal of the carbohydrate part with Endoglycosidase H results in a 135 kDa peptide [32] (Fig. 4b). Both media easily separated the digested protein from the native with approximately the same efficiency, but the multimodal media produced more narrow peaks (Fig. 4a). The digested peaks also proved to be narrower than those of the native protein sample as there is only one conformation present.

In agreement with the previous results obtained from RNases, pH 4.0 proved to be the most optimal pH value for separating the invertase variants. When using butyl or phenyl media, no significant pH effects were observed (data not shown).

DNA is a frequent contamination during protein purification and it is often highly desirable to remove the last traces of nucleic acids, usually by a chromatography or filtration step. The last peak at 32 CV in Fig. 3b is dsDNA (probably chromosomal) as confirmed by a PicoGreen fluorimetric assay. In none of the experiments DNA was retained on conventional HIC media. However, probably

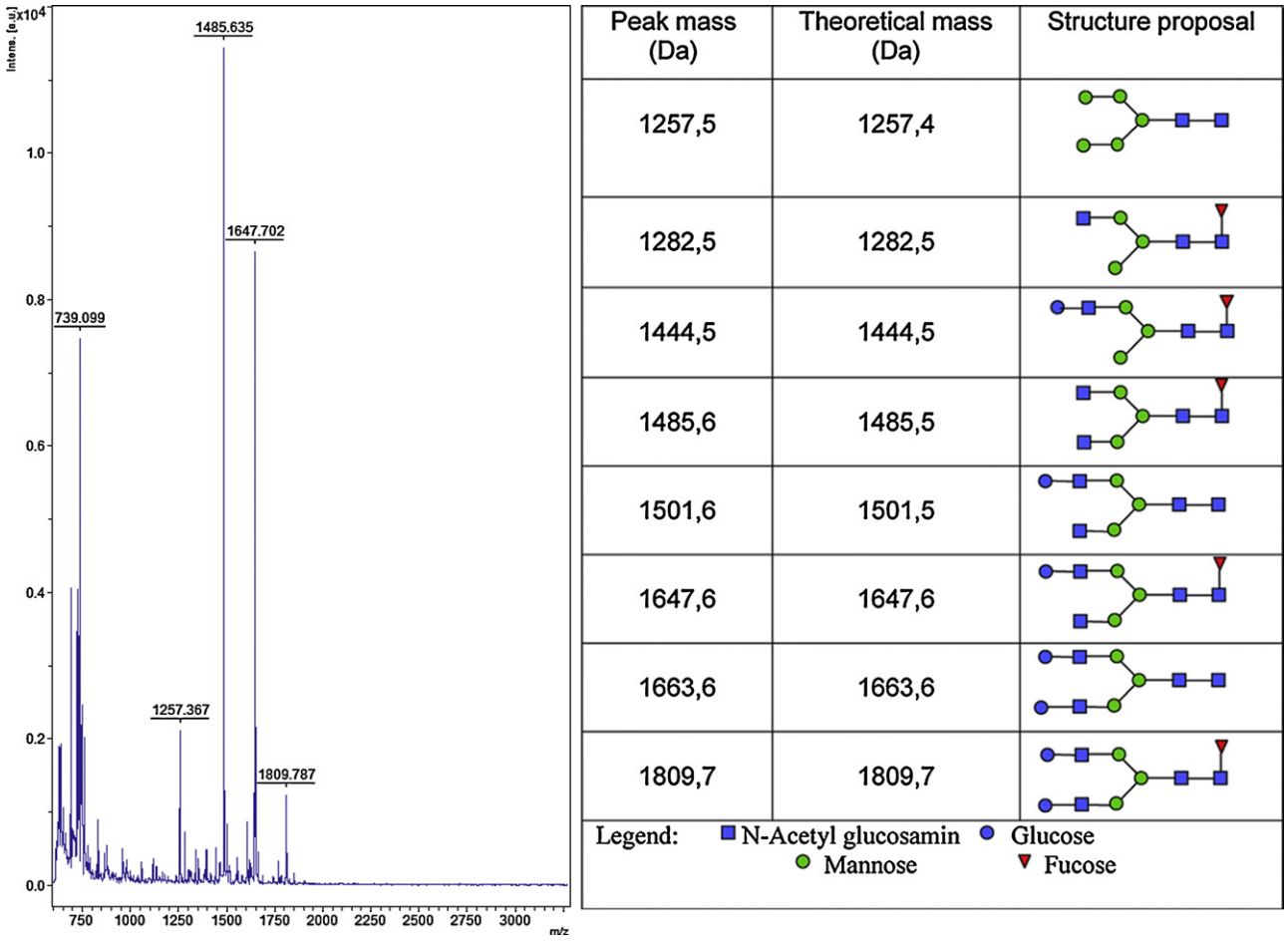

Fig. 8. MALDI spectra of mAb Y showing the glycosylation state of the protein and the sequence of the glycans.

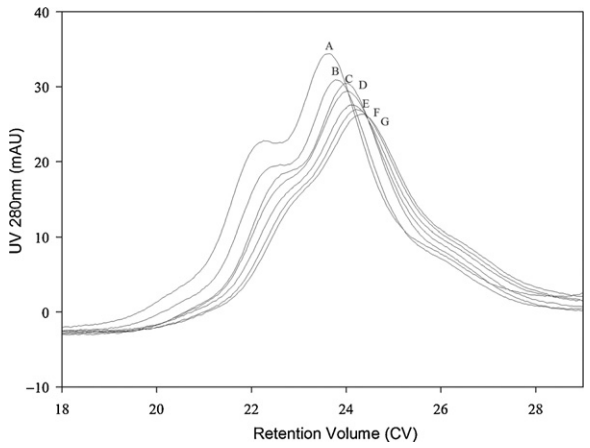

Fig. 9. PNGase digestion of mAb Y. Samples were collected at different time points during the reaction. (A) 0 min, (B) 10 min, (C) 30 min, (D) 60 min, (E) 120 min, (F) 180 min and (G) 240 min, respectively.

due to its ionic character, this separation becomes feasible on pH HIC.

3.3. Studying conformational changes in monoclonal antibodies

All human antibodies contain a single conserved glycosylation site at Asn^{297} on the constant region of the two heavy chains (Fig. 5). Previous studies have clearly demonstrated that the effector functions of IgGs are dependent on small differences in the composition of these two carbohydrate moieties and that these functions can be completely inactivated when they are removed [33–35].

Monoclonal antibody Y was deglycosylated enzymatically with PNGase F to produce mAb Y′. As shown in Fig. 6a this generated a retention shift of 0.7 CV on the multimodal media and also changed the shape of the peak significantly (Fig. 6). The peak shoulder on the left side became less pronounced and the peak resembled that of the naturally unglycosylated antibody X (Fig. 7). The glycosylation states of mAb Y and X were verified by MALDI TOF mass spectroscopy (Fig. 8). The monoclonal antibody Y showed a complex carbohydrate pattern and produced 8 distinct peaks that could be assigned as glycans, while the control mAb X exhibited none (data not shown). The core structures of the glycans are well preserved in a biantennary core shape consisting of $Man_3GlcNAc_2$–Asn^{297}, as is common in IgGs, and the size of the glycans only varies between 1257 and 1809. For comparison, mAbs Y and Y′ were also injected on a traditional phenyl HIC column where neither the retention shift nor change of peak shape occurred (Fig. 6).

In order to better monitor the enzymatic deglycosylation process, samples of mAbs were collected during the entire 4 h reaction time (Fig. 9). The most significant changes occurred within the first hour, after which the peak continued to shift in retention but the shape was unchanged. This clearly demonstrates the potential of using HIC as a simple first tool to determine the degree of protein glycosylation. The two sugar residues on the antibody keeps the Cγ2 domain separated in a more flexible "open" conformation which is beneficial for Fc-receptor binding [36]. When they are removed the antibody enters a "closed" state. The multimodal HIC media is thus able discriminate between the proteins of different glycosylation states.

4. Conclusions

The behaviour of a pH responsive multimodal HIC media in regard to the glycosylation patterns of proteins was examined. The media itself showed remarkable responsiveness to small variations in pH, which made it possible to change the chromatographic conditions to the current isolation issue. Optimal glycoprotein separation was obtained at pH 4 for the media used.

The possibility to study glycosylation patterns on proteins using the multimodal HIC media was also evaluated. By designing the chromatography media to allow for multiple interaction types, small structural changes in conformation could be detected, e.g. in glycosylated and deglycosylated human monoclonal antibodies. In addition, it was possible to monitor the enzymatic deglycosylation reaction by collecting samples at different time intervals and comparing retention of the eluted peaks.

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References

[1] L. Krishnamoorthy, L.K. Mahal, ACS Chem. Biol. 4 (2009) 715.
[2] D.P. Gamblin, E.M. Scanlan, B.G. Davis, Chem. Rev. 109 (2009) 131.
[3] R. Abès, J.-L. Teillaud, Pharmaceuticals 3 (2010) 146.
[4] B.J. Scallon, S.H. Tam, S.G. McCarthy, A.N. Cai, T.S. Raju, Mol. Immunol. 44 (2007) 1524.
[5] S. Udiavar, A. Apffel, J. Chakel, S. Swedberg, W.S. Hancock, E. Pungor, Anal. Chem. 70 (1998) 3572.
[6] C.F. Goochee, T. Monica, Biotechnology (N Y) 8 (1990) 421.
[7] R.G. Werner, K. Kopp, M. Schlueter, Acta Paediatr. Suppl. 96 (2007) 17.
[8] S. Elliott, T. Lorenzini, S. Asher, K. Aoki, D. Brankow, L. Buck, L. Busse, D. Chang, J. Fuller, J. Grant, N. Hernday, M. Hokum, S. Hu, A. Knudten, N. Levin, R. Komorowski, F. Martin, R. Navarro, T. Osslund, G. Rogers, N. Rogers, G. Trail, J. Egrie, Nat. Biotechnol. 21 (2003) 414.
[9] T.U. Gerngross, Nat. Biotechnol. 22 (2004) 1409.
[10] A.M. Sinclair, S. Elliott, J. Pharm. Sci. 94 (2005) 1626.
[11] B.M. Brena, F. Batista-Viera, L. Ryden, J. Porath, J. Chromatogr. 604 (1992) 109.
[12] P.M. Rudd, I.G. Scragg, E. Coghill, R.A. Dwek, Glycoconjugate J. 9 (1992) 86.
[13] M. Kullolli, W.S. Hancock, M. Hincapie, J. Sep. Sci. 31 (2008) 2733.
[14] M. Madera, Y. Mechref, M.V. Novotny, Anal. Chem. 77 (2005) 4081.
[15] G. Gaza-Bulseco, A. Bulseco, C. Chumsae, H. Liu, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 862 (2008) 155.
[16] T. Graniér, R. Bhikhabhai, J. Zhou, GE Discov. Mat. Mag. (2007) 21.
[17] K.R. Anumula, Anal. Biochem. 220 (1994) 275.
[18] K. Becker, M. Grey, L. Bulow, J. Chromatogr. A 1215 (2008) 152.
[19] K. Becker, E. Hallgren, E. Carredano, R. Palmgren, L. Bulow, J. Mol. Recogn. 22 (2009) 104.
[20] K. Becker, J. Van Alstine, L. Bulow, J. Chromatogr. A 1202 (2008) 40.
[21] J. Van Alstine, C. Larsson, Å. Rudstedt, R. Palmgren, in: International Patent WO2004/082801, 2004.
[22] C. de Las Heras Alarcon, S. Pennadam, C. Alexander, Chem. Soc. Rev. 34 (2005) 276.
[23] A. Kumar, A. Srivastava, I.Y. Galaev, B. Mattiasson, Prog. Polym. Sci. 32 (2007) 1205.
[24] Y. Xiao, T.T. Jones, A.H. Laurent, J.P. O'Connell, T.M. Przybycien, E.J. Fernandez, Biotechnol. Bioeng. 96 (2007) 80.
[25] P. Maharjan, M.T. Hearn, W.R. Jackson, K. De Silva, B.W. Woonton, J. Chromatogr. A 1216 (2009) 8722.
[26] L. Zhou, J. Wan, X. Cao, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 878 (2010) 1025.
[27] R.L. Williams, S.M. Greene, A. McPherson, J. Biol. Chem. 262 (1987) 16020.
[28] D. Fu, L. Chen, R.A. O'Neill, Carbohydr. Res. 261 (1994) 173.
[29] J.M. Prien, D.J. Ashline, A.J. Lapadula, H. Zhang, V.N. Reinhold, J. Am. Soc. Mass Spectrom. 20 (2009) 539.
[30] S. Fexby, L. Bulow, Trends Biotechnol. 22 (2004) 511.
[31] N.P. Neumann, J.O. Lampen, Biochemistry 6 (1967) 468.
[32] A.L. Tarentino, T.H. Plummer Jr., F. Maley, J. Biol. Chem. 249 (1974) 818.
[33] Y. Kaneko, F. Nimmerjahn, J.V. Ravetch, Science 313 (2006) 670.
[34] J.N. Arnold, M.R. Wormald, R.B. Sim, P.M. Rudd, R.A. Dwek, Annu. Rev. Immunol. 25 (2007) 21.
[35] M. Nose, H. Wigzell, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 6632.
[36] S. Krapp, Y. Mimura, R. Jefferis, R. Huber, P. Sondermann, J. Mol. Biol. 325 (2003) 979.