Glycoconj J (2015) 32:625–634
DOI 10.1007/s10719-015-9608-4

ORIGINAL ARTICLE

Glycome characterization of immunoglobulin G from buffalo (*Bubalus bubalis*) colostrum

L. S. Mamatha Bhanu[1] · M. Amano[2] · S.-I. Nishimura[2] · H. S. Aparna[1]

Received: 30 March 2015 / Revised: 24 June 2015 / Accepted: 29 June 2015 / Published online: 4 August 2015

Abstract Immunoglobulin G (IgG) is a major glycoprotein in ruminant colostrum. First day buffalo colostrum protein was purified on Sephadex G-100 and its mass was determined by MALDI-TOF as 147.848 KDa. The PMF data of protein subunits revealed its homology to IgG, which was supported by the identification of peptide sequences LLIYGATSR and VYNEYLPAPIVR corresponding to light and heavy chains of IgG by CID MS/MS analysis. The N-glycan microheterogeneity was established based on chemoselective glycoblotting technique with the identification of high mannose, neutral complex/hybrid and sialylated complex/hybrid glycans. A complete structural assignment of 54 N-linked oligosaccharides were identified and the ratio of sialyl oligosaccharides was found to be higher compared to neutral saccharides. The fucosylation observed in more than 20 oligosaccharides, high mannose and trisialyl oligosaccharides were present in diminutive amount. The high non-fucosyl and sialyl oligosaccharides in buffalo colostrum IgG provide ample scope for its utilization in targeted therapies to elicit effective ADCC and anti-inflammatory responses.

Keywords *Bubalus bubalis* · Colostrum · Glycoblotting · Immunoglobulin G · MALDI-TOF MS

Abbreviations

MALDI-TOF	Matrix-assisted laser desorption/ ionization-time of flight
LC-MS/MS	Liquid chromatography–mass spectrometry
PNGase F	Peptide -N-Glycosidase F
PHM	1-propanesulfonic acid 2-hydroxyl-3-myristamido
DTT	Dithiothreitol
IAA	Iodoacetic acid CID, Collision induced dissociation
PMF	Peptide mass fingerprinting
ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement-dependent cytotoxicity

Introduction

Colostrum, the lacteal secretion vital for mammalian newborn is a complex physiological fluid loaded with free oligosaccharides, glycoproteins, gangliosides, phagocytes, antimicrobial and immune-enhancing components which are unique compared to normal milk [1, 2]. Apart from normal constituents of milk, colostrum contains immune-enhancing factors like growth factors, immunoglobulins, putative permeability fraction and fraction containing enzymes, proteins and peptides [3]. The unique components of colostrum like free oligosaccharides and glycoconjugates are known to offer resistance to enzymatic digestion in the gastrointestinal tract and have the ability to inhibit the localized adherence of enteropathogens to the digestive tract of the neonates [4, 43]. The levels of oligosaccharides are much higher in buffalo milk compared to other

Electronic supplementary material The online version of this article (doi:10.1007/s10719-015-9608-4) contains supplementary material, which is available to authorized users.

✉ H. S. Aparna
ls.mamatha@gmail.com

[1] Department of Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, India

[2] Faculty of Advanced Life Sciences, Hokkaido University, Sapporo 001-0021, Japan

domestic animals milk [5]. Since, the primary role of glycoconjugates is to provide protection against pathogens, they are being used as neutraceuticals in functional foods [6].

Immunoglobulin G (IgG) is a principal globular protein (~70 %) present in colostral whey with remarkable effects against infectious enteric and respiratory diseases [7, 1]. The Ig concentration is very high 54.0 mg/ml in buffalo colostrum [8] and four Igs classes IgG, IgA1, IgA2 and IgM have been identified in lacteal secretions [9]. Igs are the family of glycoproteins with 82–96 % of protein and 4–18 % of carbohydrate [10]. Earlier investigations indicated the presence of only two subclasses of IgG in bovine colostrum (IgG1 and IgG2).

IgG molecule is a multifunctional glycoprotein that binds antigens (pathogens) especially to form immune complexes that activate effector mechanism such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) through their interaction either with lymphocyte receptors on effector cells such as natural killer cells or with the C1q component of complement resulting in the clearance and destruction [11]. Several studies have shown that non-fucosylated IgG can exhibit an increased ADCC [12] and antiviral activities [13] in a mouse model system [14, 15]. But, the immunosuppressive property of IgG is largely dependent on sialylation of the Fc N-glycans [16]. Thus glycosylation on IgG is known to play a very crucial role in eliciting effective immune responses.

The significance of glycoconjugates and bioactive peptides in neonatal health care has led to extensive exploitation of bovine colostrum. Since buffalo (*Bubalus bubalis*) is the major milking mammal contributing 63 % milk production in India, purification and characterization of IgG was carried out to explore the glycan assembly to establish its structure-function relationship. In the present study, we have attempted to isolate, purify and characterize IgG from buffalo colostrum by employing MALDI-TOF-MS and CID MS/MS. The N-glycome analysis is going to be performed by adopting chemoselective glycoblotting technique. In addition, the preferential lectin binding activity of IgG will also be established using human erythrocytes.

Materials and methods

Milk sample

Buffalo colostrum sample was collected on the first day of lactation locally by a single animal of about 500 ml and kept frozen at −20 °C until further analysis.

Isolation and purification of immunoglobulin G (IgG)

Colostrum (100 ml) was used to isolate whey proteins as described earlier [17]. The whey proteins were subjected to 60 % ammonium sulphate fractionation followed by centrifugation at 3,000 rpm for 30 min at 4 °C [18]. The pellet was suspended in water, dialyzed extensively against water and lyophilized. The sample (60 % pellet) was then fractionated on Sephadex G-100 column (1.5×90 cm) using 0.1 M NaCl in deionized water. Protein fractions (2 ml/10 min) collected were measured at 280 nm and peak fractions were pooled, dialyzed, lyophilized and stored at −20 °C until further analysis.

MALDI-TOF analysis of intact protein

MALDI-TOF analysis was performed on an Autoflex II mass spectrometer (Bruker Daltonics, Germany) using the matrix 2, 5-dihydroxybenzoic acid (DHB; 20 mg/ml) in acetonitrile containing 0.1 % trifluoroacetic acid. The analyte (0.5 μl) and matrix (0.5 μl) were thoroughly mixed on the MALDI plate. For protein analysis, ions were analyzed in the linear mode after acceleration at 20 kV and with an extraction delay of 500 ns. Mass spectra were acquired by scanning the m/z range from 1,000 to 24,000 at 100 ms/scan.

In-gel trypsin digestion

The purified protein bands resolved on SDS-PAGE were excised separately and subjected to in-gel trypsin digestion. Briefly, the excised gel was crushed into small pieces, transferred to sterile Eppendorff and destained by repeated washing with 50 mM ammonium bicarbonate (NH_4HCO_3) buffer. The gel pieces were rehydrated in DTT (10 mM), NH_4HCO_3 (100 mM, 30 min) and alkylated with iodoacetamide (50 mM) in NH_4HCO_3 (100 mM). The sample was dehydrated using acetonitrile and vacuum dried. Trypsin digestion was performed by incubating the dried gel pieces overnight with trypsin (Promega Porcine trypsin, 20 μl of 20 ng/μl stock solution containing 50 mM NH_4HCO_3) at 37 °C. The tryptic peptides were extracted from gel using 50 % acetonitrile containing 1 % TFA. The extraction step was repeated (×4) and the pooled peptides were dried in a speed-vac [19].

MALDI- TOF MS analysis

Tryptic peptides were analyzed by MALDI-TOF tandem mass spectrometer in positive ion reflector mode using Ultra flex TOF/TOF (Bruker Daltonics, Bremen, Germany) equipped with nitrogen laser (337 nm). The enzyme digest samples were mixed and equal volumes of sample and saturated matrix solution (2, 5-dihydroxybenzoic acid in 50 % acetonitrile containing 0.1 % TFA) were mixed and spotted on a MALDI plate and the spectra were recorded in the linear mode using Bruker Daltonics FLEX control software [20].

LC-MS/MS analysis

HPLC workflow was performed on an Agilent system (HP1100 Series) with detection set at 226 nm. The tryptic peptides were separated on C-18 column (4.6×150 mm; ZORBAX RX-C18, 5 μm, Agilent) using a linear gradient of acetonitrile containing 0.1 % formic acid (5–95 %) in 55 min at a flow rate of 200 μl/ min. LC was interfaced directly with a ion trap (HCT ULTRA ETD II, Bruker Daltonics, Germany) mass spectrometer equipped with two Octapole followed by an ion trap and a separate hexapole next to negative chemical ionization chamber (nCI). LC-MS/MS data were processed using Esquire data analysis software, version 4.0 and .mgf file extracted from data analysis software were analyzed by Mascot MS/MS ion search database.

Release of N- glycans

The N-glycans were released from IgG purified by gel filteration as described [21]. Briefly, the sample (350 μg) was mixed with 0.4 % PHM (15 μl), 0.33 M NH_4HCO_3 (30 μl) and 120 mM DTT (10 μl) and reduced at 60 °C for 30 min. The alkylation was carried out by adding 123 mM IAA (20 μl) followed by incubation in dark at room temperature. The mixture was then treated with trypsin (400 U) and incubated at 37 °C for 3 h. The reaction was terminated by heat inactivation at 90 °C for 10 min. The sample was finally incubated with PNGase F (2 U) at 37 °C overnight and vacuum dried.

Chemoselective glycoblotting

The glycoblotting was performed as previously described [22]. In brief, the vacuum dried N- linked glycans constituted in distilled water (18 μl) were mixed with internal standard (20–50 pmoles) and aliquoted on to a MultiScreen Solvinert filter plate containing Blotglyco H beads (250 μl, 10 mg/ml) followed by addition of 2 % AcOH/ACN (180 μl). The plate was incubated at 80 °C for 60 min and the beads were sequentially washed with 200 μl, 2 M guanidine HCl, distilled water and 1 % triethylamine/CH_3OH. The plate was then incubated with 100 μl 10 % Ac2O/ CH_3OH at room temperature for 30 min and washed step-wise in 200 μl 10 mM HCl, CH_3OH and dioxane. The esterification of sialic acids was performed in presence of 100 μl of 100 mM MTT/dioxane with incubation at 60 °C for 60 min with subsequent washings in 200 μl dioxane, CH_3OH and distilled water. The N-glycans were labelled as BOA derivatives by adding 20 μl of 50 mM and 20 mM BOA, respectively, and 2 % AcOH/ACN (180 μl) and incubated at 80 °C for 60 min. The BOA labelled glycans were recovered by washing the beads with distilled water (100 μl) and vacuum dried. The total labelled glycans (1 μl) mixed with DHB: DHB Na (9:1, 2 μl)) was spotted on the MALDI plate and subjected for MALDI-TOF MS.

Analysis of N -glycans

The BOA labelled N-glycans were analyzed by MALDI-TOF mass spectrometer in positive ion reflector mode using Auto flex III TOF/TOF (Bruker Daltonics, Bremen, Germany). The spectra were recorded in a linear mode using FLEX CONTROL 3.0 soft ware.

The MALDI-TOF MS data was analyzed using FLEX ANALYSIS 3.0 software. The N-glycans were identified by database search using http://www.expasy.ch/tools/glycomod; http://glycosuitedb.expasy.org and http://jcggdb.jp. The MS/ MS analysis was performed with the abundant dual structural assignment peaks.

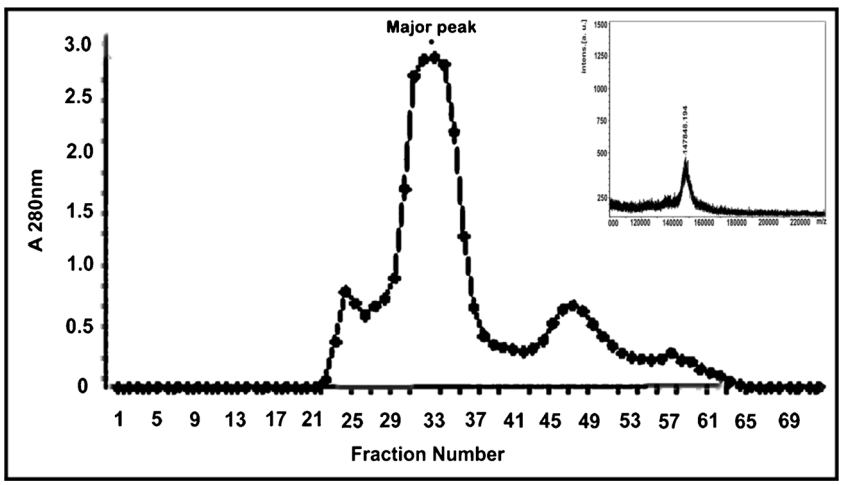

Fig. 1 Separation of Whey Proteins on Sephadex G-100 column (1.5×90 cm) using 0.1 M NaCl in deionized water with flow rate 2 ml/10 min. Inset: MALDI-TOF MS of intact protein

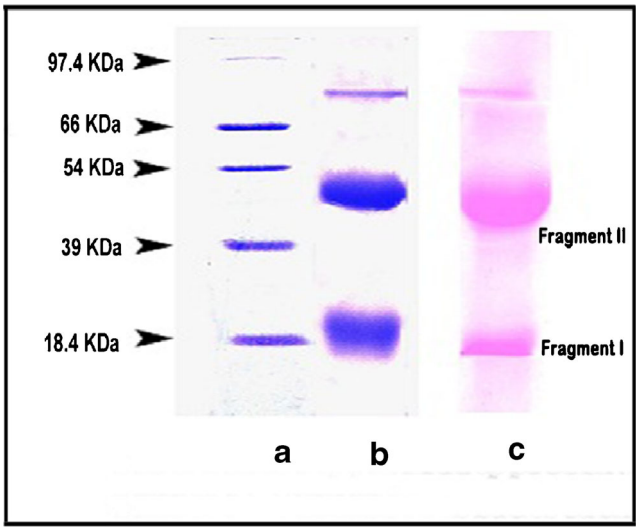

Fig. 2 Seperation of second (major) peak on SDS-PAGE. **a** Markers **b** Coommassie stain **c** PAS stain

Results and discussion

Isolation and characterization of Immunoglobulin G (Ig G)

The whey proteins isolated from first day of lactation sample of buffalo colostrum with an average yield of 18 % was subjected to ammonium sulphate fractionation (60 %). The pellet fraction was purified on Sephadex G-100. As shown in Fig. 1, the major peak fraction subjected to native and SDS-PAGE. The native PAGE (data not shown) confirmed the homogeneity of the purified protein and also its glycoprotein nature. The exact mass of the protein as determined by MALDI-TOF MS was 147.848 kDa (Fig. 1, Inset). The SDS-PAGE analysis revealed the subunits composition of the pure protein as 49 and 25 kDa (Fig. 2). Employing similar strategy we have previously purified a major whey protein, β-lactoglobulin from buffalo colostrum [23].

In order to establish the identity of the protein, the protein bands excised from SDS-PAGE were subjected for in-gel trypsin digestion. The resultant peptide mass fingerprint (PMF) of the subunits (Fig. 3; Tables 1 and 2) obtained after MALDI-TOF analysis was searched against non-redundant NCBI through Matrix Science-MASCOT search engine.

The Mascot search results revealed the identity of the purified protein as immunoglobulin G with fragment 1 (25 kDa) showing 82 % sequence homology to *Homo sapiens* immunoglobulin light chain variable region (Supplimentary file 1) while, fragment II (49 kDa) having 90 % sequence homology to *Orytologous cruniculus* immunoglobulin heavy chain V-D-J region (Supplimentary file 2). Because of limited information on the PMF of IgG either from buffalo or bovine milk/colostrum in the database, the results obtained in the present study rely to human and rabbit IgG. However, very recently gene coding for (39KDa) Fc fragment of IgG from buffalo has been uploaded to NCBI (Gene ID: 102400212). Various analytical approaches based on either separation techniques or immune assays are employed for the purification and quantitation of IgG from colostrum and milk samples [24–31]. Although the available methods can be exploited for the purification of IgG from either serum or milk, the method developed in the present investigation is simple for the rapid purification of colostrum or milk IgG with highest purity.

LC- MS/MS analysis

During the present study, LC- ESI- MS/MS analysis was performed with CID capability to derive the sequence

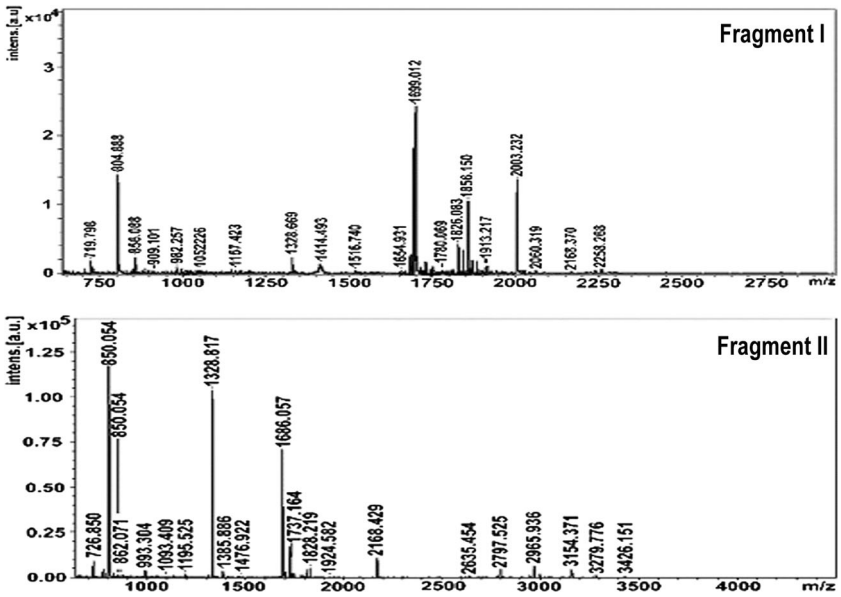

Fig. 3 Peptide mass fingerprint of purified protein (Fragment I) & (Fragment II)

Table 1 Peptide mass fingerprint data of buffalo colostrum IgG light chain

Start-End	Observed	Mr (expt)	Mr (calc)	Delta	M	Peptide
5–32	2996.1170	2995.1097	2994.4645	0.6452	0	K.ESEGGLFKPTGTLTLTCTVSGFSLSSYR.V
33–63	3426.1510	3425.1437	3424.6364	0.5073	1	R.VSWVRQAPGNGLEYIGISSGDSTYYASWAK.S
38–63	2797.5250	2796.5177	2797.2871	−0.7694	0	R.QAPGNGLEYIGYISSGDSTYYASWAK.S
71–80	1117.5210	1116.5137	1116.6139	−0.1002	0	R.NTNLNTVTLK.M
81–96	1780.2270	1779.2197	1778.7968	0.4230	0	K.MTSLTAADTATYFCAR.G
97–112	1695.6510	1694.6437	1693.8100	0.8337	0	R.GYPGYSNNIWGPGTLV,-

information of the tryptic peptides of IgG. The identification of double charged ion pairs with m/z -497^{2+} and 664^{2+} corresponding to light and heavy chains, respectively, facilitated the detection of peptide sequences of IgG. The .mgf file for m/z -497^{2+} corresponding to fragment I yielded LLIYGATSR (Fig. 4) sequence, which was in compliance to IgG Kappa chain V–III region NG9 from *Homo sapiens* (Supplimentary file 3). Similarly, the .mgf file for m/z -664^{2+} corresponding to fragment II yielded peptide sequence VYNEGLPAPIVR was in homology to immunoglobulin γ heavy chain constant region from *Cervus lelaphus hispanicus* (Supplimentary file 4). Liquid chromatography-mass spectrometry (LC-MS) is currently the most powerful analytical tool for structural characterization of therapeutic antibodies due to its superior resolution, sensitivity and accuracy. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become a promising platform for quantifying therapeutic proteins including antibody molecules in biological samples [32–34].

N-glycome of IgG

The majority of therapeutic proteins including immunoglobulin G and A are subject to post translational modifications, which can affect the therapeutic properties since content of specific sugar types like fucose and sialic acid can greatly affect the efficacy of the biopharmaceutical molecule. Since IgG is heavily glycosylated, the identification and characterization of glycans, as well as understanding their biological significance, is of increasing importance in the fields of biomedical and pharmaceutical research.

Upon proteomic characterization of IgG based on PMF and LC-MS/MS analyses, quantization of total glycome was performed by adopting chemoselective glycoblotting technique coupled to MALDI-TOF MS analysis. Quantitation of individual glycans was performed using an internal standard prior to glycoblotting. The monosaccharide compositions of neutral, sialyl and high mannose oligosaccharides of N-glycans of IgG was achieved (Fig. 5, Table 3).

A sum of 54 N-glycans was identified in the colostrum IgG sample, which was classified based on their structural complexity. As shown in Fig. 6a, IgG was highly sialylated with as high as 30 oligosaccharides, 19 neutral and 5 high mannose saccharides. Among sialyl oligosaccharides mono, and disialyl glycans were in equal proportions with trisialyl oligosaccharides being the least containing either *N*-glycolyl (NeuGc) or *N*-acetylneuraminic acids (NeuAc) or both (Fig. 6c). Interestingly, the content of non-fucosylated (31) glycans was found maximum among the characterized N-glycans. The fucosylation while observed in more than 20 oligosaccharides, monofucosyl oligosaccharides were high (19) compared to di-(2) and trifucosyl (2) oligosaccharides (Fig. 6b). The major oligosaccharides were found to be $(Hex)_2$ $(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$ and $(Hex)_2$

Table 2 Peptide mass fingerprint data of buffalo colostrum IgG heavy chain

Start-End	Observed	Mr (expt)	Mr (calc)	Delta M	Peptide
1–11	1022.3440	1021.3367	1020.5087	0. 8280 0	-, SASASLGDSVK. L
12–18	878.1220	877.1147	876.4487	0. 6660 0	K. LTCNLTR
19–34	1830.0370	1829.0297	1827.9380	1. 0917 1	R. GHSSYAIWLRQQPGK. G
30–37	868.0730	867.0657	866.4722	0. 5935 1	R. QQPGKGPR. Y
35–41	881.0210	880.0137	879.4636	0. 5501 1	K. GPRYLMK. V + Oxidation (M)
38–50	1544.7430	1543.7357	1544.7293	−0. 9936 1	R. YLMKVNNDGSYNK. G
42–50	1009.2840	1008.2767	1009.4465	−1. 1697 0	K. VNNDGSYNK. G
42–50	1011.2750	1010.2677	1009.4465	0. 8213 0	K. VNNDGSYNK. G
51–57	729.7790	728.7717	728.3453	0. 4264 0	K. GDGIPDR. F
51–67	1693.8690	1692.8617	1693.7656	−0.9039 1	K. GDGIPDRFSGSSSGAER. Y
86–103	1810.0360	1809.0287	1807.9357	1. 0931 0	K. TWGTGIEVFGGGTTLTVL. -

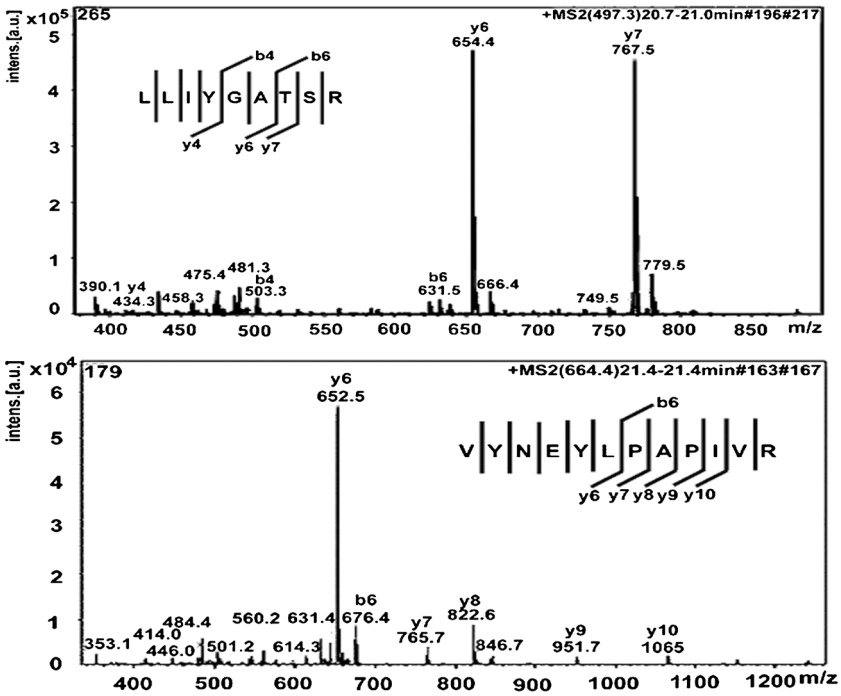

Fig. 4 LC-MS/MS analysis of IgG Fragment I of $[M + H]^{2+}$ −497.3 & Fragment II ions of $[M + H]^{2+}$ −664.4

$(HexNAc)_2$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$, which accounted for 43 % of the total N-glycans. In order to confirm the structural assignments of IgG N-glycome with the identical masses (m/z −2,335, 2,397, 2,540 and 2,556), MS/MS analysis was performed. Since the concentration of these glycans was low (2–18 pmoles), the diagnostic ions generated could not give confirmatory structures for all the glycans except for m/z −2, 335 with $(Hex)_3$ $(HexNAc)_2$ $(NeuAc)_1$+ $(Man)3(GlcNAc)_2$ (Supplimentary file 5) and m/z −2,556 with $(Hex)_2$ $(HexNAc)_2$ $(Deoxyhexose)_1$ $(NeuGc)_2$ + $(Man)_3$ $(GlcNAc)_2$ (Supplimentary file 6) structures, respectively (Supplementary file 5 and 6). These results indicated that high sialylation and non-fucosylation being obvious in buffalo colostrum IgG was also the general feature observed in bovine milk N-glycome but, human milk was found to possess more fucosylated and sialylated oligosaccharides [35, 36] Although some structurally similar N-glycans were identified in bovine colostrum in analogy to buffalo colostrum IgG [37].

In the light of this information, buffalo colostrum IgG containing higher order of non-fucosyl N-glycans along with high mannose oligosaccharides can be successfully exploited for the development of novel antibodies to elicit effective ADCC activity [38]. Terminal Gal, GlcNAc and Man residues affect C1q binding and CDC activity whereas, terminal NeuAc, Man, core Fuc and bisecting GlcNAc residues are known to affect FCγRIIIa binding and ADCC [39]. Owing to structural characterization of sialyl oligosaccharides from colostrum IgG, they are implicated as potential therapeutic

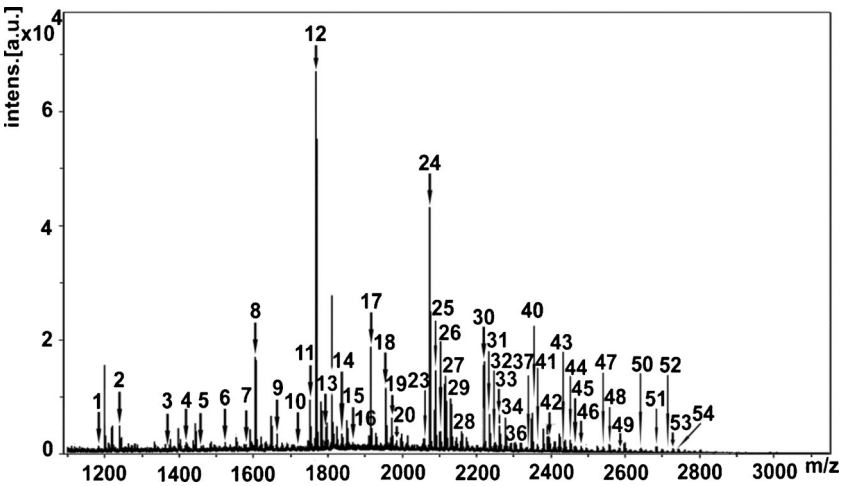

Fig. 5 MALDI-TOF-MS analysis of IgG N-glycans

Table 3 N-linked glycans of buffalo colostrum IgG

Peak No	m/z	Composition
1	1184.357	$(Hex)_3$ $(HexNAc)_2$ $(Deoxyhexose)_1$
2	1241.255	$(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$
3	1362.286	$(Hex)_2$ + $(Man)_3(GlcNAc)_2$
4	1403.299	$(Hex)_1$ $(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$
5	1444.338	$(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$
6	1524.33	$(Hex)_3$ + $(Man)_3(GlcNAc)_2$
7	1590.384	$(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
8	1606.387	$(Hex)_1$ $(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$
9	1686.365	$(Hex)_4$ + $(Man)_3(GlcNAc)_2$
10	1708.389	$(Hex)_1$ $(HexNAc)_1$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
11	1752.423	$(Hex)_1$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
12	1768.426	$(Hex)_2$ $(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$
13	1793.441	$(HexNAc)_3$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
14	1848.413	$(Hex)_5$ + $(Man)_3(GlcNAc)_2$
15	1850.401	$(HexNAc)_4$ + $(Man)_3(GlcNAc)_2$
16	1851.476	$(HexNAc)_1$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
17	1914.469	$(Hex)_2$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
18	1955.489	$(Hex)_1$ $(HexNAc)_3$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
19	1971.485	$(Hex)_2$ $(HexNAc)_3$ + $(Man)_3(GlcNAc)_2$
20	2010.451	$(Hex)_6$ + $(Man)_3(GlcNAc)_2$
21	2013.488	$(Hex)_1$ $(HexNAc)_1$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
22	2045.523	$(Hex)_1$ $(HexNAc)_1$ $(NeuGc)_2$ + $(Man)_3(GlcNAc)_2$
23	2057.484	$(Hex)_1$ $(HexNAc)_2$ $(Deoxyhexose)_1$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
24	2073.503	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
25	2089.514	$(Hex)_2$ $(HexNAc)_2$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
26	2101.52	$(Hex)_1$ $(HexNAc)_3$ $(Deoxyhexose)_2$ + $(Man)_3(GlcNAc)_2$
27	2117.544	$(Hex)_2$ $(HexNAc)_3$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
28	2142.506	$(HexNAc)_4$ $(Deoxyhexose)_2$ + $(Man)_3(GlcNAc)_2$
29	2191.511	$(Hex)_2$ $(HexNAc)_1$ $(NeuAc)_1$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
30	2219.542	$(Hex)_2$ $(HexNAc)_2$ $(Deoxyhexose)_1$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
31	2235.549	$(Hex)_3$ $(HexNAc)_2$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
32	2247.546	$(Hex)_1$ $(HexNAc)_3$ $(Deoxyhexose)_3$ + $(Man)_3(GlcNAc)_2$
33	2251.523	$(Hex)_3$ $(HexNAc)_2$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
34	2276.541	$(Hex)_2$ $(HexNAc)_3$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
35	2288.54	$(HexNAc)_4$ $(Deoxyhexose)_3$ + $(Man)_3(GlcNAc)_2$
36	2301.556	$(HexNAc)_4$ $(Deoxyhexose)_1$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
37	2317.563	$(Hex)_1$ $(HexNAc)_4$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
38	2320.585	$(Hex)_2$ $(HexNAc)_4$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$
39	2333.531	$(Hex)_1$ $(HexNAc)_4$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
40	2348.576	Internal Standard
41	2378.57	$(Hex)_2$ $(HexNAc)$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
42	2394.551	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_1$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
43	2397.522	$(Hex)_4$ $(HexNAc)_2$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
44	2410.516	$(Hex)_2$ $(HexNAc)_2$ $(NeuGc)_2$ + $(Man)_3(GlcNAc)_2$
45	2422.593	$(Hex)_2$ $(HexNAc)_3$ $(Deoxyhexose)_1$ $(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$
46	2454.556	$(Hex)_3$ $(HexNAc)_3$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
47	2524.601	$(Hex)_2$ $(HexNAc)_2$ $(Deoxyhexose)_1$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
48	2540.602	$(Hex)_3$ $(HexNAc)_2$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
49	2556.594	$(Hex)_2$ $(HexNAc)_2$ $(Deoxyhexose)_1$ $(NeuGc)_2$ + $(Man)_3(GlcNAc)_2$

Table 3 (continued)

Peak No	m/z	Composition
50	2654.609	$(Hex)_1$ $(HexNAc)_4$ $(NeuGc)_2$ + $(Man)_3(GlcNAc)_2$
51	2683.635	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_3$ + $(Man)_3(GlcNAc)_2$
52	2699.634	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_2$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
53	2727.662	$(Hex)_2$ $(HexNAc)_3$ $(Deoxyhexose)_1$ $(NeuAc)$ + $(Man)_3(GlcNAc)_2$
54	2743.621	$(Hex)_3$ $(HexNAc)_3$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
55	2759.660	$(Hex)_3$ $(HexNAc)_3$ $(NeuAc)_1$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$

agents in glycan interaction associated with some of the diseases like cancer, rheumatoid arthritis and influenza [13]. Furthermore the presence of $Man_9GlcNAc_2$, a high mannose type of N-glycan often recognized by dendritic cell surface receptor during viral infection suggests its functional utility in developing antiviral inhibitors [40]. Two glycopeptide fragments of N-linked sugar chains linked to either Asn-55 or Asn-215 residue were obtained by digestion of the protein with Achromobacter protease I [41]. HPLC analysis showed that bovine lactoferrin-a was found to consist of fucose, galactose, and *N*-acetylgalactosamine in addition to mannose and *N*-acetylglucosamine [42]. The combined use of two novel techniques, chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry allows for both facile purification and precise analysis of common oligosaccharides and glycopeptides from native glycoproteins.

Conclusions

Since there was no recent work on buffalo colostrum IgG the present study was carried out to unravel the structural complexity and determine functional ability of IgG. The IgG with bound N-glycans to Asn297 is known to play key roles in mediating IgG stability and undergo characteristic glycosylation changes during variety of inflammatory conditions and in aging. In addition, both human milk and bovine milk oligosaccharides have received a lot of interest mainly for their biological efficacy as prebiotics, anti-inflammatory and immune modulators and as a possible source of sialic acid for the growth and development of nervous system. Hence, the present study was carried out to unravel the structural complexity and determine functional ability of IgG. The IgG purified from buffalo colostrum was characterized based on

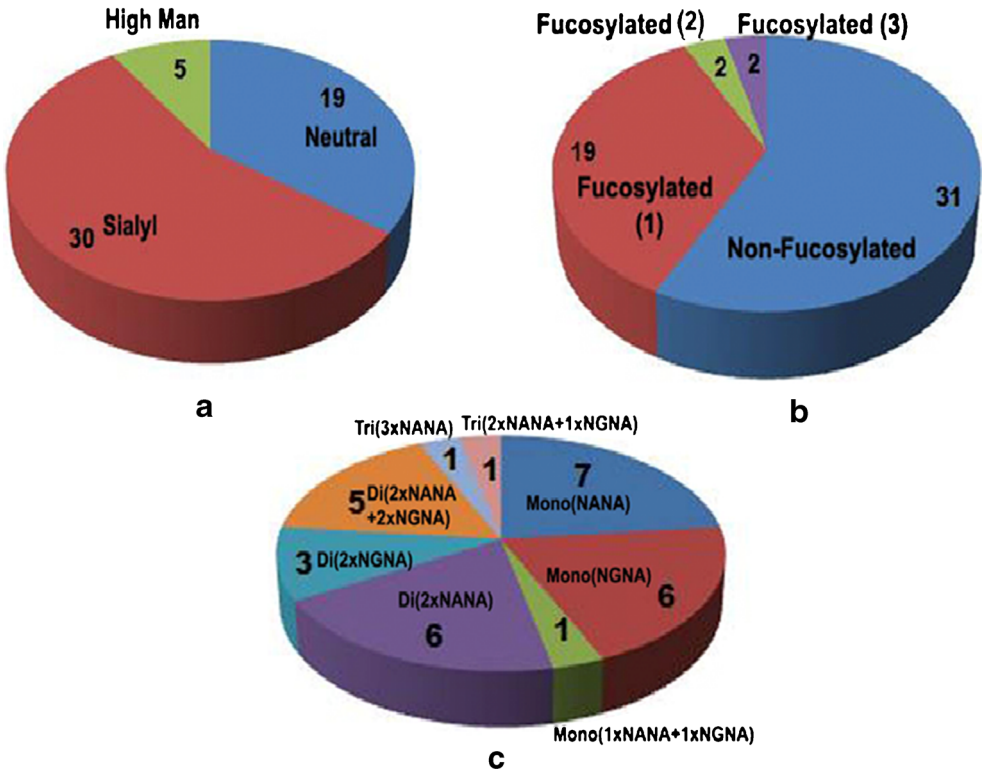

Fig. 6 Pie charts showing the relative abundance of N-glycans (**a**) Total N-glycans (**b**) Sialylated N- glycans (**c**) Fucosylated N-glycans in buffalo colostrum IgG

MALDI-TOF MS and LC-MS/MS analyses. The glycome analysis revealed the presence of 54 N-glycans with only two O-glycans. The study was comprehensive showing N-glycan microheterogeneity with the presence of high mannose, neutral complex/hybrid and sialylated complex/hybrid N-glycans. Like bovine milk, buffalo colostrum IgG also contained both NeuAc and NeuGc unlike human milk, which possesses only NeuAc. The presence of NeuGc was confirmed by MS/MS analysis. Antibodies are currently the most heavily consumed protein therapeutics for human therapy, which can have profound impact on its functional utility in therapeutic interventions.

Acknowledgments HSA thanks INSA-JSPS for the award of overseas associateship and Mamatha Bhanu thanks University Post-graduate cell for the award of fellowship. Authors also thank protemics facility, IISc, Bangalore.

Conflict of interest The authors declare no commercial or financial conflict of interest.

Funding This work was supported by the Indian National Science Academy (INSA) and the Japan Society for the Promotion of Science (JSPS) fund.

References

1. Pramod, G., Gill, H.S.: Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br. J. Nutr. **84**, 69–74 (2000)
2. Aurelia, P., Cristian, C., Camelia, R., Vioara, M., Gheorghe, M.: The study of the main parameters quality of buffalo milk. Afr. J. Microbiol. Res. **10**, 201–206 (2009)
3. Antonius, M.: In vivo antimicrobial and an-tiviral activity of components in bovine milk and colostrum involved in non-specific defence. Br. J. Nutr. **84**, 127–134 (2000)
4. Uruakpa, O., Ismond, M.A.H., Akobundu, E.N.T.: Colostrum and its benefits: a review. Nutr. Res. **22**, 755–767 (2002)
5. Martinez-Ferez, A., Rudloff, S., Guadix, A., Cordula, A.H., Pohlentz, G., Boza, J.B., Guadix, E.M., Kunz, C.: Goat's milk as a natural source of lactose-derived oligosaccharides: isolation by membrane technology. Int. Dairy J. **16**, 173–181 (2006)
6. Colarow, L., Turini, M., Tencherg, S., Berger, A.: Characterization and biological activity of gangliosides in buffalo milk. Biochim. Biophys. Acta **1631**, 94–106 (2003)
7. Bulter, J.E.: Bovine immunoglobulins: an augmented review. Vet. Immunol. Immunopath. **4**, 43–152 (1983)
8. Dang, A.K., Kapila, S., Purohit, M., Singh, C.: Changes in colostrum of Murrah buffaloes after calving. Trop. Anim. Health. Pro. **41**, 1213–1217 (2008)
9. Kulkarni, B.A.: Immunoglobulins of the Indian buffalo. V. Changes in colostrum milk and neonatal calf serum immunoglobulins in early lactation. Indian J. Biochem. Biophys. **18**, 28–31 (1981)
10. Goodman, W.: Immunoglobulin structure and function. In: Stites, D.P., *et al.* (eds.) Basic and clinical Immunology, p. 27. Lang. Med. Pub., Los Altos, CA (1987)
11. Arnold, N., Wormald, M.R., Sim, R.B., Rudd, P.M., Dwek, R.A.: The impact of glycosylation on the biological function and structure of human immunoglobulins. Ann. Rev. Immunol. **25**, 21–50 (2007)
12. Zeitlin, L., Pettitt, J.J., Scully, C.C., Bohorova, N.N., Do, D.K., Pauly, M.M., Hiatt, A.A., Ngo, L.L., Steinkellner, H.H., Whaley, K.J.K., Olingerb, G.G.: Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. Proc. Natl. Acad. Sci. **108**, 20690–20694 (2011)
13. Kai-Ting, S., Robert, M.A.: Antibody glycosylation and Inflammation. Antibodies **2**, 392–414 (2013)
14. Nimmerjahn, F., Ravetch, J.V.: Divergent Immunoglobulin G subclass activity through selective Fc receptor binding. Sci. **310**, 1510–1512 (2005)
15. Junttila, T.T., Parsons, K., Olsson, C., Lu, Y., Xin, Y., Theriault, J., Crocker, L., Pabonan, O., Baginski, T., Meng, G., Totpal, K., Kelley, R.F., Sliwkowski, M.X.: Superior in vivo efficacy of afucosylated trastuzumab in the treatment of HER2-amplified. Breast Canc. Canc. Res. **70**, 4481–4489 (2010)
16. Kaneko, Y., Nimmerjahn, F., Ravetch, J.V.: Anti-inflammatory activity of Immunoglobulin G resulting from Fc sialylation. Sci. **313**, 670–673 (2006)
17. Aparna, H.S., Salimath, P.V.: Purification of an antigenic glycopeptide from buffalo colostrum. J. Food Sci. Technol. **38**, 450–452 (2001)
18. Kanade, S.R., Rao, D.H., Hegde, R.N., Gowda, L.R.: The unique enzymatic function of field bean (Dolichos lablab) D-galactose specific lectin: a polyphenol oxidase. Glycoconj. J. **26**, 535–545 (2009)
19. Rosenfeld, J., Capdevielle, Guillemot, C., Ferrara, P.: In-gel digestion for internal sequence analysis after one-or two-dimensional gel electrophoresis. Anal. Biochem. **203**, 173–179 (1992)
20. Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P., Franzen, J., Holle, A.: A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal. Bioanal. Chem. **376**, 952–965 (2003)
21. Kita, Y., Muira, Y., Furukawa, J., Nakano, M., Shinohara, Y., Ohno, M., Takimoto, A., Nishimura, S.-I.: Quantitative glycomics of human whole serum glycoproteins based on the standardized protocol for liberating N-glycans. Mol. Cell. Proteomics **6**, 1437–1445 (2007)
22. Furukawa, J., Shinohara, Y., Kuramoto, H., Miura, Y., Shimaoka, H., Kurogochi, M., Nakano, M., Nishimura, S.-I.: Comperhensive approach to structural and functional glycomics based on chemoselective glycoblotting and sequential tag conversion. Anal. Chem. **80**, 1094–1101 (2008)
23. Rohit, A.C., Aparna, H.S.: Characterization of β-lactoglobulin from buffalo colostrum and its possible interaction erythrocyte lipocalin-interacting-membrane-receptor (LIMR). J. Biochem. **150**, 279–288 (2011)
24. McKinney, M., Parkinson, A.: A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J. Immunol. Methods **96**, 271–278 (1987)
25. Premysl, K., Rodney, J.B., William, H.S.: Chromatographic purification of immunoglobulin G from bovine milk whey. J. Chromatograph. A **673**, 45–53 (1994)
26. Didier, L., Alain, O.: Bovine immunoglobulin G, β-lactoglobulin, α-lactalbumin and serum albumin in colostrum and milk during the early postpartum period. J. Dairy Res. **66**, 421–430 (1999)
27. Korhonen, H., Marnila, P., Gill, H.S.: Milk immunoglobulins and complement factors. Br. J. Nutr. **84**, 75–80 (2000)
28. Gapper, L.W., Copestake, D.E., Otter, D.E., Indyk, H.E.: Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements a review. Anal. Bioanal. Chem. **389**, 93–109 (2007)
29. Elke, S., Leitner, B., Ryan, M.M., Elizabeth, H.D., Farhat, K., John, W., Evelina, A.: Evaluation of immunoglobulin purification methods and their impact on qualitative and yield of antigen-specific antibodies. Malarial J. **7**, 129–138 (2008)
30. Menegatti, S., Hussain, M., Naik, A.D., Carbonell, R.G., Rao, B.M.: mRNA display selection and solid-phase synthesis of Fc-binding cyclic peptide affinity ligands. Biotechnol. Bioeng. **110**, 857–870 (2013)

31. Tong, F., Lin, D.Q., Yuan, X.M., Yao, S.J.: Enhancing IgG purification from serum albumin containing feedstock with hydrophobic charge-induction chromatography. J. Chromatogr. A **1244**, 116–122 (2012)
32. Ezan, E., Bitsch, F.: Critical comparison of MS and immunoassays for the bioanalysis of therapeutic antibodies. Bioanalysis **1**, 1375–1388 (2009)
33. Wu, H., Chen, S., Shortreed, M.R., Kreitinger, G.M., Yuan, Y., Frey, B.L., Zhang, Y., Mirza, S., Cirillo, L.A., Olivier, M., Smith, L.M.: Sequence-specific capture of protein-DNA complexes for mass spectrometric protein identification. PLoS One. **6**(10): e26217 (2011)
34. Mesmin, C., Cholet, S., Blanchard, A., Chambon, Y., Azizi, M., Ezan, E.: Mass spectrometric quantification of AcSDKP-NH2 in human plasma and urine and comparison with an immunoassay. Rapid Commun. Mass Spectrom. **26**, 163–172 (2012)
35. Nwosu, C.C., Aldredge, D.L., Lee, H., Lerno, L.A., Zivkovic, A.M., German, J.B., Lebrilla, C.B.: Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. J. Proteome Res. **11**, 2912–2924 (2012)
36. Nongluk, S., Sachiko, K., Hirokazu, Y., Hiroaki, H., Shin-ichi, N., Keita, Y., Ito, H., Hisashi, N.J., Koichi, K., Yasuo, S.: Bovine milk whey for preparation of natural N-glycans: structural and quantitative analysis. Open Glysosci. **5**, 41–50 (2012)
37. Takimori, S., Shimaoka, H., Furukawa, J., Yamashita, T., Amano, M., Fujitani, N., Takegawa, Y., Hammarström, L.I., Kacskovics, Shinohara, Y., Nishimura, S.: Alteration of the N-glycome of bovine milk glycoproteins during early lactation. FEBS J. **278**, 3769–3781 (2011)
38. Robert, A., Fredrik, W., Jeffrey, V.R.: Novel roles for the Fc glycan. Ann. N.Y. Acad. Sci. **1253**, 170–180 (2012)
39. Hodoniczky, J., Zheng, Y.Z., James, D.C.: Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. Biotechnol. Prog. **21**, 1644–1652 (2005)
40. Menon, S., Rosenberg, K., Graham, S.A., Ward, E.M., Taylor, M.E., Drickamer, K.: Binding-site geometry and flexibility in DC-SIGN demonstrated with surface force measurements. Proc. Natl. Acad. Sci. U. S. A. **106**, 11524–11529 (2009)
41. Sato, T., Takio, K., Kobata, A., Greenwalt, D.E., Furukawa, K.: Site-specific glycosylation of bovine butyrophilin. J. Biochem. **117**, 147–157 (1995)
42. Wei, Z., Nishimura, T., Yoshida, S.: Presence of a glycan at a potential N-glycosylation site, Asn-281, of Bovine Lactoferrin. J. Dairy Sci. **83**, 683–689 (2000)
43. Urashima, T., Taufik, E., Fukuda, K., Asakuma, S.: Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Biosci. Biotechnol. Biochem. **77**, 455–466 (2013)