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Screening neutral and acidic IgG N-glycans by high density electrophoresis

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IgG carries bi-antennary N-linked glycans which differ in degrees of galactosylation, core fucosylation and bisecting N-acetyl glucosamine. The majority of these are non-sialyated closely related neutral structures which can be resolved by HPLC analysis, but which are difficult to separate in techniques such as fluorophore-coupled carbohydrate electrophoresis. Derivatisation with the singly charged fluorophore, 2-amino benzoic acid and separation in gels with a 30% monomer content in tris/glycine buffer enabled separation of neutral glycans. In particular, agalactosyl glycans with either a core fucose substitution or bisecting N-acetyl galactosamine could be resolved. Good separation of mono- and di-galactosylated glycans was also achieved with this system. It was shown that IgG can be separated from serum by size-exclusion and anion exchange chromatography with minimal contamination, with complete glycan release accomplished by the enzyme peptide-N-glycosidase F (*F. meningosepticum*). This method of resolving IgG glycans could be used to monitor patients in which glycosylation changes may have a diagnostic value, as in rheumatoid arthritis. It could also be used to monitor recombinant IgG glycosylation where routine screening is required in the biotechnology industry.

Keywords: **carbohydrate PAGE, IgG glycosylation, biantennary, neutral glycan**

Introduction

A method which could routinely screen IgG glycosylation would provide useful biochemical information for monitoring diseases such as rheumatoid arthritis where there is an established, measurable difference in glycan profile when compared to healthy individuals. Furthermore, it would be a valuable addition to current techniques for the quality control of recombinant glycoproteins. IgG contains N-linked, biantennary (1,3 and 1,6 linked to tri-mannosyl core) oligosaccharides with heterogeneity at the non-reducing terminus. The conserved N-glycosylation sites are located on the heavy chains of the antibody and account for 85% of the total glycosylation [1]. The Fc region is invariably glycosylated at Asn 297 on each of the Cγ2 domains. There is variable glycosylation on the Fab portion dependent upon the occurrence of Asn-X-Ser/Thr sites in the hypervariable regions. Rheumatic diseases are associated with changes in IgG oligosaccharide profiles [2]. For example, rheumatoid arthritis (RA) is associated with hypogalactosylation [3]. There are several ways in which hypogalactosylation could potentially contribute to the pathology of RA. For example immune complexes with rheumatoid factor tend to bind with higher affinity to agalactosylated IgG [4] which may lead to deposition in the joint. In addition, the interaction between mannose binding protein and exposed N-acetylglucosamine structures on agalactosylated IgG can activate complement [5].

Currently, there are several methods for determining IgG glycosylation profiles. Originally, gel permeation chromatography on Biogel P4 which allowed the detection of changes in galactosylation was used. However, it has limited resolution and is not suitable for detailed analysis; nor can it be used for sialylated glycans. Chromatographic techniques such as HPLC or mass spectrometry can give the required resolution and sensitivity, but they involve access to expensive equipment and require expert interpretation of the data and are, therefore, not ideally suited to screening analysis. Lectins have been used to detect agalactosyl glycans [6], however, the final data does not provide a profile, rather a ratio of glycans with differing levels of galactosylation. This method is not definitive as it depends on the reactivity of the lectin used and the experimental conditions.

A technique which is ideal for profiling complex mixtures of glycans is high-density PAGE of fluorophore-labelled reducing saccharides [7]. The fluorophore labels

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which have been used previously such as 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS, [7]) and 2-aminoacridone [8] are able to resolve charged glycans. However, with neutral glycans, such as the heterogeneously galactosylated saccharides of IgG, the relatively high molecular weight and the strong negative charge from the sulphonyl groups of a label such as ANTS may give poor resolution of the glycans. We have used the much smaller, single charged fluorophore, 2-aminobenzoic acid, to resolve the neutral glycans of IgG.

Materials and methods

All chemicals were Analar grade and purchased from Sigma Chemical Co. unless stated otherwise.

Preparation of serum

Blood samples were allowed to clot and spun at 6000 rpm, 4 × 750ml Windsheild rotor (Mistral 3000, MSE) for 15 min. The serum was removed and stored at −20 °C.

IgG purification

IgG was purified from serum in a single operation using a column of coarse Sephadex G-25 (Sigma) and an anion exchange resin DE52 (Whatman) [6]. 12.0 ml of Sephadex were layered on to 1.0 ml of anion exchange resin. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0 and 250 µl of serum diluted 1:1 with 10 mM sodium phosphate buffer pH 7.0 was applied. After the column was washed with 2.5 ml of 10 mM, pH 7.0 sodium phosphate buffer, 0.5 ml fractions were collected. IgG was eluted in these fractions. The peak fraction containing IgG was determined by a bicinchinonic acid protein assay (Pierce). The purity of the peak fraction containing IgG was determined by 12% acrylamide SDS-PAGE prepared with protogel reagents (National Diagnostics) and compared to a similar method for purifying IgG which uses 20 mM potassium phosphate, pH 7.2 as elution buffer [6]. The yield and subclass distribution of IgG purified by both methods was determined in triplicate by nephelometry and ELISA (using antibodies to IgG1, IgG2, IgG3 and IgG4) [9] respectively.

Comparison between enzymatic and hydrazinolysis methods for glycan release from IgG

A preparation of normal human serum IgG (from 250 µl of serum) was divided into three aliquots and two of these were treated with endoglycosidase: peptide-N-glycosidase F (*F. meningosepticum*) (PNGaseF; 6 Units/sample in incubation buffer supplied by Oxford GlycoSciences) for 6 or 24 hr. The third aliquot served as a control and contained no PNGaseF. Release of glycans from IgG pre and post treatment with PNGaseF was performed on a GlycoPrep 1000 (Oxford GlycoSciences) in the N* programme mode (reaction for 5 hr at 95 °C) following exhaustive dialysis of the protein solution in a Slide-a-Lyzer™ dialysis system (Pierce) against 0.1% TFA for 96 hr at 4 °C. The collected glycan pool was evaporated to dryness by rotary evaporation (Eyela, 30 °C) and re-dissolved in 200 µl water before labelling with 2-amino benzamide (2-AB labelling kit, Oxford GlycoSciences) and analysis by HPLC. Further preparations of IgG glycans were prepared by incubating IgG (100 µl, 5 mg/ml protein) at 37 °C for 24 h with 6 U of PNGaseF.

HPLC analysis of IgG glycans

Separation on a 4.6mm × 250 mm amide bonded silica HPLC column (GlycoSep N, Oxford GlycoSciences) was performed according to the method described by Guile *et al.* [10] using a gradient of 35–50% of 0.5 M ammonium formate pH 4.4 in acetonitrile, over 2–80 min at a flow rate of 0.4 ml/min.

Glycan labelling

Protein was removed using a micro-concentrator with 10 kDa molecular weight cut off (Sartorius). The glycans were labelled with 2-aminobenzoic acid (2-AA labelling kit, Oxford GlycoSciences [11]).

Glycan resolution

Fluorophore-labelled IgG glycans (750 pmoles) were loaded onto 30% polyacrylamide/19% ethylene glycol, Tris-Acetate, pH 7.0 gel and run at a constant current of 15 mA/gel (max. 800 V) until the dye front had just run off the end of the gel. Either 50 mM Tris-Tricine buffer pH 8.0, 50 mM Tris-TAPS buffer pH 8.0 or 50 mM Tris-Glycine buffer pH 8.0 were compared as running buffers for resolving a series of glycan standards: di-sialylated-, galactosylated biantennary glycan (G2S2); di-sialylated-, galactosylated biantennary glycan, core substituted with fucose (G2fS2); asialo-, agalacto-, biantennary glycan (G0); asialo-, agalacto-, biantennary glycan with core substituted fucose (G0f); asialo-, agalacto-, biantennary glycan with core substituted fucose and with bisecting N-acetyl glucosamine (G0fb); asialo-, galactosylated, biantennary glycan (G2); asialo-, galactosylated, biantennary glycan with bisecting N-acetyl glucosamine (G2b); asialo-, galactosylated, biantennary glycan with core substituted fucose (G2f); asialo-, galactosylated, biantennary glycan with core substituted fucose and with bisecting N-acetyl glucosamine (G2fb) (Oxford GlycoSciences). The relative positions of the resolved glycan standards were determined by running a homopolymer dextran ladder (Oxford GlycoSciences) in one of the PAGE lanes.

Quantitative analysis of glycan bands

The gel was placed on a UG1 filter (Oxford GlycoSciences) on top of a mid range (312 nm) UV transilluminator and was imaged on a GAS7000 gel documentation system (UVItec). Background light was minimised by Wratten 2A and Wratten 11 filters (Sigma). The bands were detected using UVIPhoto version 97.04 for Windows (UVI-Pro) and quantified with UVIband version 97.04 for Windows.

Analysis of sample variance

The process of IgG purification, glycan release, labelling and analysis was repeated 11 times on different days with two operators using the same sample of serum. The coefficient of variance was calculated for 6 of the resolved bands. The coefficient of variance was calculated by dividing the standard deviation by the mean. The effect of dilution on fluorescence band volume was determined by diluting a stock glycan solution and loading 4µl of each serially diluted sample.

Results

The effect of ionic strength on yield and purity of IgG purified from serum.

The yield and purity of serum derived IgG samples were compared to samples prepared by a similar but more common method [6] in which the eluant buffer is 20 mM potassium phosphate pH 7.2, instead of 10 mM sodium phosphate pH 7.0. The average yield of IgG purified with 10 mM ionic strength buffer was between 60–73% and for 20 mM buffer it was between 84–96%. Although the yield of IgG was greater using 20 mM potassium phosphate pH 7.2, there was a higher level of contaminating proteins. In particular, the glycoprotein transferrin can be detected, which is a cause for concern as the IgG glycans prepared would therefore be mixed with glycans derived from transferrin. Figure 1 shows a SDS-PAGE of 4 samples of IgG purified by each method. The transferrin band is clearly visible at 70 kDa in each of the samples purified using 20 mM buffer, whereas it is absent from the samples purified with 10 mM buffer. The subclasses of IgG purified by each method was determined to evaluate whether the method adopted for this study purified a representative sample of all the IgG subclasses. The same proportion of IgG1 and IgG2 was seen in serum and IgG purified by each method (Fig. 2). However, there was a loss in the relative proportion of IgG4 particularly in the IgG sample purified by 10 mM sodium phosphate pH 7.0 (Fig. 2), although this is only a small proportion of the total IgG.

Comparison between chemical and enzymatic methods for release of IgG glycans

The release of IgG glycans by PNGaseF was analysed by HPLC following labelling with 2-amino benzamide (2-AB). Figure 3 shows that there was little difference in the profile of glycans released by PNGase-F or by automated hydrazinolysis, both in terms of the type of glycans or the relative amounts of each glycan. No glycans were released by hydrazinolysis from the residual protein following the PNGase-F digestion. Furthermore, it was found that the release of glycans was complete after 6 hours incubation with PNGase-F as judged by treatment of the residual protein with hydrazine.

Identification of glycan bands resolved on carbohydrate electrophoresis

The biantennary glycans released from IgG were run on high density carbohydrate PAGE and shown to resolve into four major bands (Fig. 4, lane 1). In order to confirm the glycan identity of each band, a pool of IgG glycans was

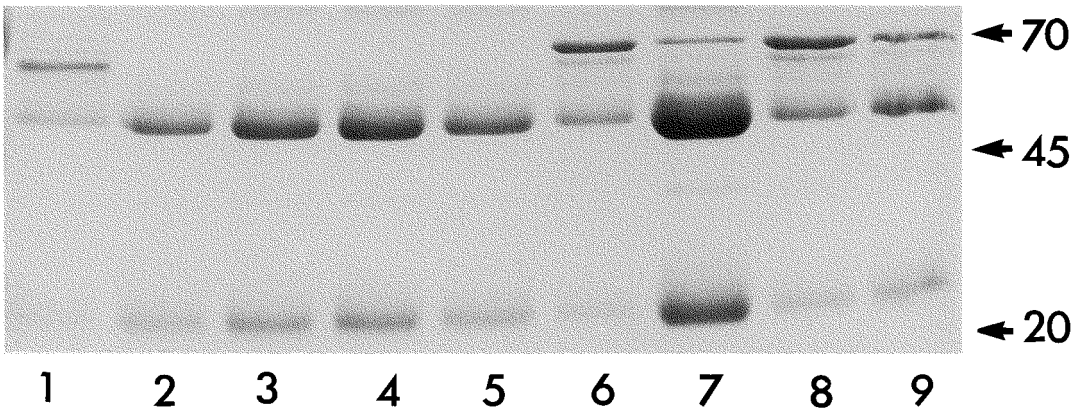

Figure 1. Comparison of two methods for purifying IgG from serum. SDS-PAGE stained with Coomassie blue for the peak fractions eluted from gel filtration/anion exchange columns used to purify IgG from serum. Lane 1 transferrin (Sigma); lane 2 IgG (Sigma); lanes 3–5 peak fractions from columns run with 10 mM Sodium phosphate pH 7.0; lanes 6–9 peak fractions from columns run with 20 mM Potassium phosphate pH 7.0. The position of the molecular weight markers in kDa is shown on the right hand side.

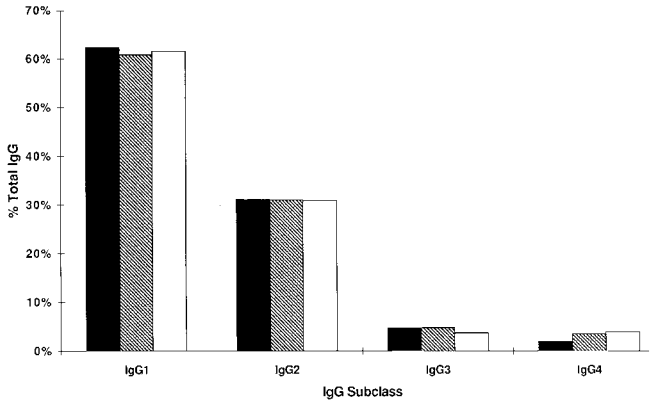

Figure 2. The subclass distribution of IgG eluted using either 10 mM Sodium phosphate pH 7.0 (black columns) or 20 mM potassium phosphate pH 7.2 (grey columns) and compared to the original serum (white columns). The subclass distribution was determined by ELISA. Standard deviations were calculated from triplicate experiments.

labelled with 2-AA and run on a normal phase amide HPLC column and fractions corresponding to each peak collected and analysed by carbohydrate PAGE. Figure 4 shows the band position on PAGE of glycans separated by HPLC. The identity of glycans in each HPLC peak were determined by a predictive HPLC method developed by Rudd *et al.* [12]. Predictive HPLC showed that the neutral glycans resolved into 3 bands on the carbohydrate gel which ran very close together. Of the three bands, the top one contained di-galactosylated structures, the middle one mono-galactosylated structures and the bottom one was agalactosylated glycans. The band that ran furthest into the gel (Fig. 4) was sialylated di-galactosylated glycan.

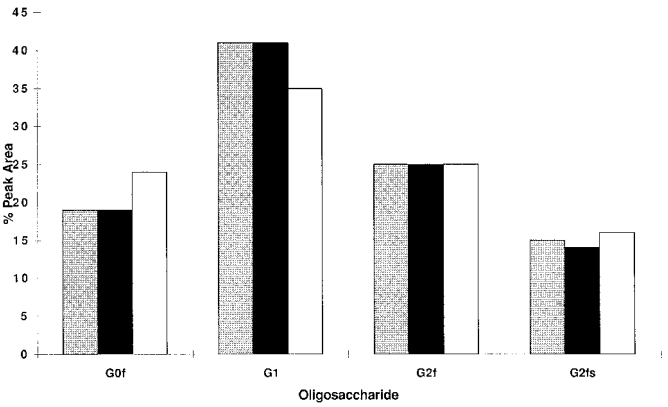

Figure 3. Comparison of two methods of releasing N-glycans from IgG. A sample of IgG was split into 3 samples. One was incubated with PNGase-F for 6 h (dotted columns), the second was incubated with PNGase-F for 24 h (black columns) and the glycans of the control sample were released by hydrazinolysis (white columns). The proportion of glycan released by each method was determined by normal phase HPLC.

The effect of running buffer counterions on PAGE resolution of IgG glycans

The resolution of nine fluorophore-labelled, standard biantennary glycans on high density PAGE is shown in Figure 5A-C. The type of counterion in the running buffer used to resolve the glycan mixture affected the number of bands resolved on the gel. Three types of running buffer were tested: Tris-Tricine (Fig. 5A); Tris-TAPS (Fig. 5B) and Tris-Glycine (Fig. 5C). The seven neutral structures resolved in the top half of the gels (Fig. 5A–C). The two di-sialylated-, di-galactosylated structures (one with core-substituted fucose) were completely resolved and separated from neutral glycans and focused in the latter part of the gels (Fig. 5A–C). Tris-Glycine was found to give the best resolution of the seven neutral biantennary structures which resolved into 5 bands (lane 1, Fig. 5C). Although it was possible to resolve the agalactosyl structures with core fucosylation or bisecting N-acetyl glucosamine, it was not possible to resolve the same variants of the di-galactosylated structure. However, the agalactosyl and the di-galactosyl structures were well resolved. With Tris-Tricine and Tris-TAPS resolving buffers (Fig. 5A and 5B respectively) the same seven neutral glycans resolved into 3 bands.

The set of nine glycan standards resolved by Tris-Glycine on carbohydrate PAGE was compared to the glycan mixture released from IgG (Fig. 6). In Figure 4 it was seen that the neutral glycans of IgG resolved in to three bands. The gel in Figure 6 was examined to see if a Tris-glycine running buffer improved the resolution of the neutral glycans. The IgG glycan bands (lanes 1 and 2, Fig. 6) were compared to lane 6 which contained the glycan standards. It was evident that G0 band was not visible and G0fb band appeared as a faint shadow between the second and third bands. This preparation of IgG glycans has a low proportion of G0 and G0fb, however, in other IgG samples the bands corresponding to G0 and G0fb were detected. The effect of transferrin contamination on the IgG glycan band profile is also shown in Figure 6. The glycans resolved in lane 1 are from IgG contaminated with transferrin (purified with 20 mM Potassium phosphate pH 7.2, Fig. 1 lanes 6–9) and lane 2 (Fig. 6) are from IgG without transferrin (purified with 10mM Sodium phosphate pH 7.0, Fig. 1, lanes 3–5). The oligosaccharide content of transferrin is shown in lane 3 (Fig. 6) and found to be mostly a di-sialylated, galactosylated biantennary glycan (standard shown in lane 5, Fig. 6). This transferrin glycan band is absent in IgG glycans purified with 10 mM Sodium phosphate pH 7.2 (lane 2, Fig. 6), whereas it can be clearly seen in the profile of IgG glycans purified with 20mM Potassium phosphate pH 7.2.

The effect of glycan band volume on the coefficient of variance

Reproducibility of the technique was investigated by analysis of IgG glycans prepared from the same batch of serum

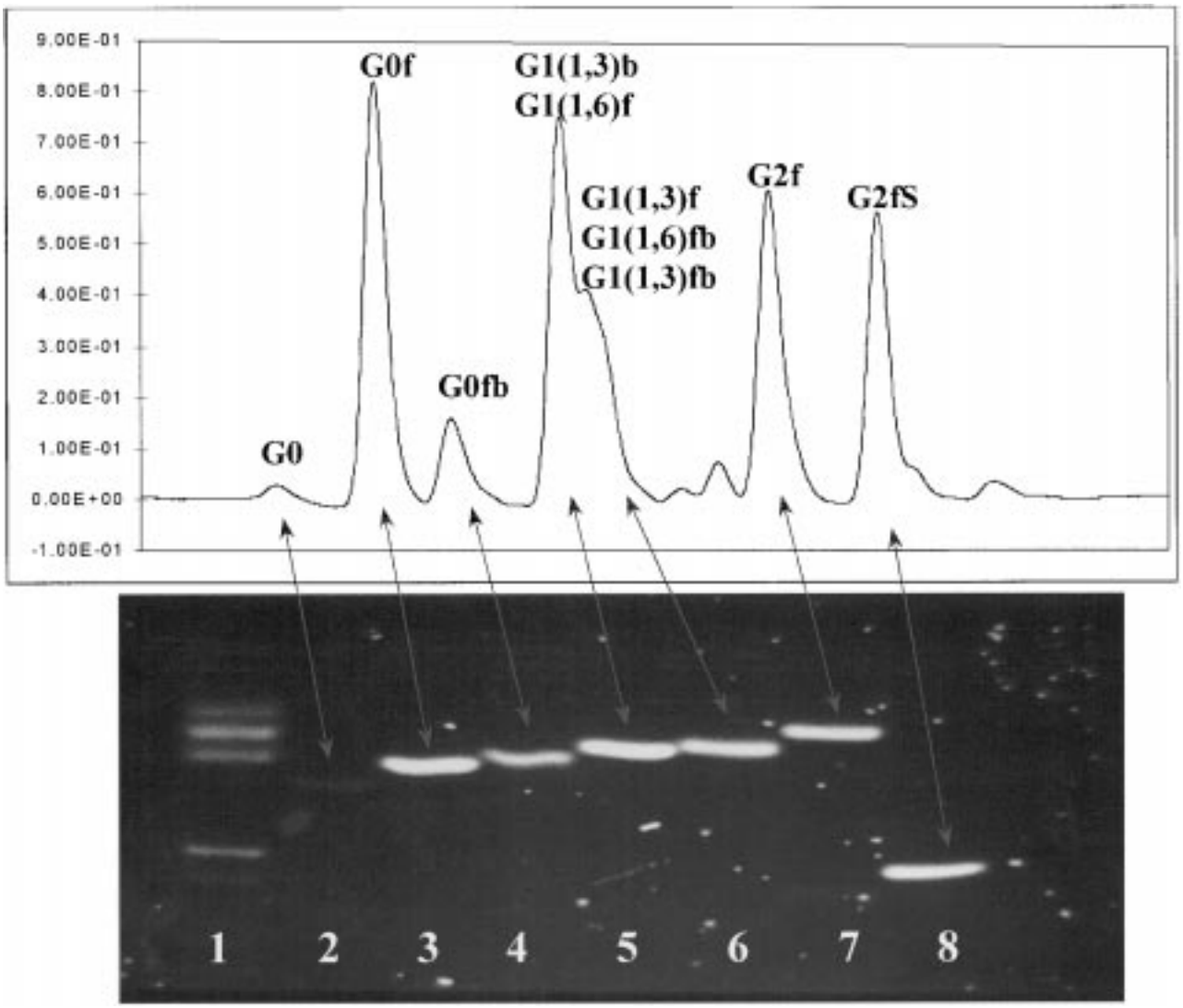

Figure 4. Peak fractions from normal phase HPLC separation of 2-AA labelled IgG N-glycans resolved on 30% polyacrylamide/19% ethylene glycol, Tris-Acetate gel, resolved by Tris-Tricine buffer. Lane 1 IgG N-glycan mixture; lane 2 peak fraction corresponding to G0; lane 3 peak fraction corresponding to G0f; lane 4 peak fraction corresponding to G0fb; lane 5 peak fraction corresponding to asialo-, galactosylated, biantennary glycan with galactose attached to 1,3 arm and with bisecting N-acetyl glucosamine (G1(1,3)b) and asialo-, galactosylated, biantennary glycan with galactose attached to 1,6 arm and with core substituted fucose (G1(1,6)f); lane 6 peak fraction corresponding to asialo-, galactosylated, biantennary glycan with galactose attached to 1,3 arm and with core substituted fucose (G1(1,3)f), asialo-, galactosylated, biantennary glycan with galactose attached to 1,6 arm and with core substituted fucose and bisecting N-acetyl glucosamine (G1(1,6)fb) and asialo-, galactosylated, biantennary glycan with galactose attached to 1,3 arm and with core substituted fucose and bisecting N-acetyl glucosamine (G1(1,3)fb); lane 7 peak fraction corresponding to G2f; lane 8 peak fraction corresponding to G2fS

in eleven separate runs of the method. A low coefficient of variance of 0.04 was obtained for more intense bands with an average of 33% of total lane fluorescence (Fig. 7). However, when the weakest bands, with an average of 2% of total lane fluorescence, were analysed the coefficient of variance was high at 0.25, so changes in the total fluorescence signal of the weak bands should be interpreted with caution. In Figure 8, serial dilutions of a glycan mixture were resolved to see if there was a linear relationship between band volume and dilution. For the 100% glycan solution (Fig. 8), approximately 2 nmoles of glycan mixture (calculated from the protein concentration and assuming 2.5 mol glycan/mol IgG) was loaded. Measurement of the fluorescence showed a linear decrease in signal correlating with glycan dilution. For example, the fluorescense band volume of the mono-sialylated-, galactosylated biantennary glycan, core substituted with fucose (G2fS) band in the 100% glycan mixture was 13229 and in the 10% glycan

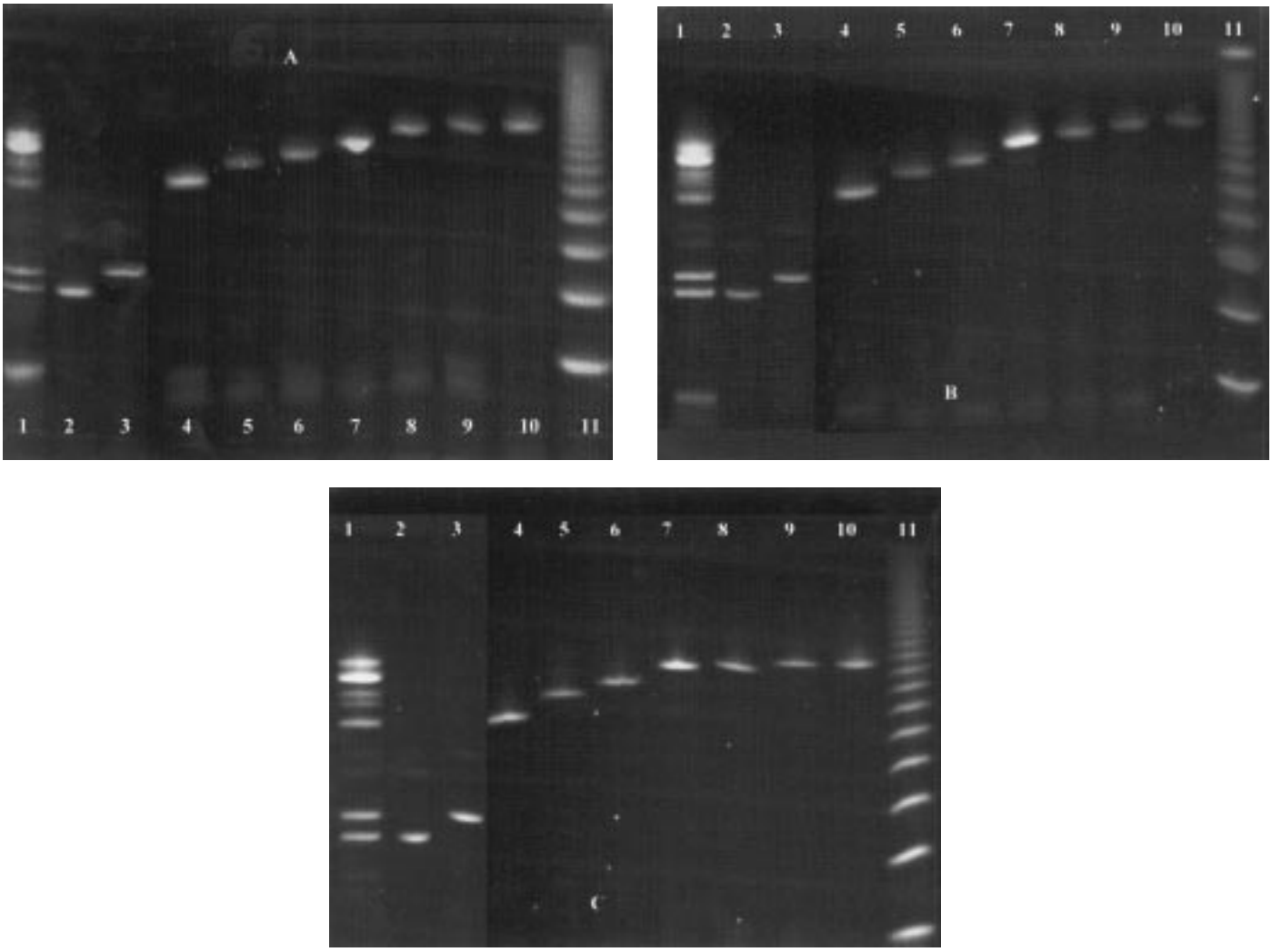

Figure 5. Identical samples run on 30% polyacrylamide/19% ethylene glycol, Tris-acetate gel at 15 mA/gel (max. 800V) with 3 different running buffers. (A) 50 mM Tris-Tricine buffer pH 8.0; (B) 50 mM Tris-TAPS buffer pH 8.0; (C) 50 mM Tris-Glycine buffer pH 8.0. Approximately 100 pmoles of each glycan standard was loaded. Lane 1 contains a mixture of all the glycan standards present in lanes 2–9; lane 2 G2S2; lane 3 G2fS2; lane 4 G0; lane 5 G0f; lane 6 G0fb; lane 7 G2; lane 8 G2b; lane 9 G2f; lane 10 G2fb; lane 11 glucose homopolymer dextran ladder.

mixture the same band had a total fluorescence signal of 1301 (Fig. 8).

Discussion

The N-linked biantennary glycans of human serum IgG have been enzymatically released from the protein and analysed by fluorophore-labelled carbohydrate electrophoresis. The method is suitable for routine, cost-effective screening of IgG glycans as it determines the relative amounts of neutral biantennary glycans. An important factor when studying a protein with low amounts of glycosylation, such as IgG, is the presence contaminating glycoproteins which may have a significant effect on analysis. In the present study, this was clearly demonstrated for transferrin which was found to be the major protein contaminant of IgG. The presence of a highly sialylated band, characteristic of transferrin but of low abundance in IgG could be seen on the gels and so a purification method which reduced its concentration to a minimum was therefore selected for this study. The method selected reduced the yield of IgG, by 24% compared to the more conventional method [6] and the subclass distribution showed less IgG4 which decreased from 4% in serum to 2% of the purified IgG. However, this small difference is unlikely to be significant in this analysis and the reduction in yield is acceptable as the contaminating protein may give misleading results. The disialylated glycan originating from transferrin was clearly visible in the glycan mixture with contaminating transferrin, but the glycan was absent from IgG glycans purified by the method adopted for our study.

It is important to demonstrate that the technique for

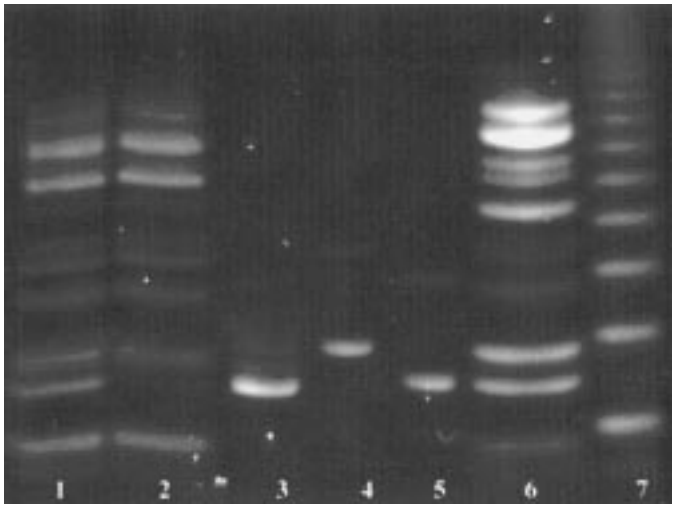

Figure 6. 2-AA labelled glycans resolved on 30% polyacrylamide/19% ethylene glycol, tris-acetate gel, resolved on Tris -Glycine buffer. Lane 1 glycans from IgG purified using 20 mM Potassium phosphate pH 7.2; lane 2 glycans from IgG purified using 10 mM Sodium phosphate pH 7.0; lane 3 glycans from transferrin; lane 4 G2fS2; lane 5 G2S2; lane 6 same as lane 1 Figure 4 (IgG N-glycan mixture); lane 7 glucose homopolymer dextran ladder.

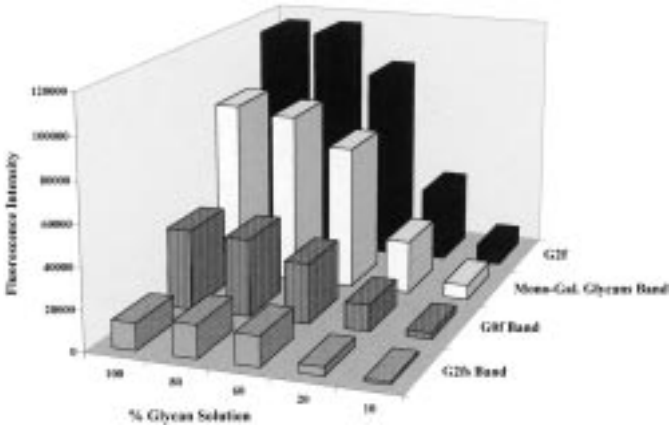

Figure 8. The effect of dilution on fluorescence signal. A stock glycan solution was serially diluted and 4 μl of each sample resolved on 30% gels. The black bars are the volumes of the band corresponding to G2f band; white bars correspond to mono-galactosylated glycans band; striped bars correspond to G0f band and dotted bars correspond to G2fS band.

glycan release is effective, as the partial or selective release of glycans could invalidate the results. The site on which IgG is glycosylated at Asn 297 is internally situated within the Fc dimer [13] and it might be expected that this would present steric hindrance to its removal by a relatively large enzyme molecule. Incomplete release of glycans by PNGaseF has been observed in some glycoproteins especially when the protein has not been denatured. However, as shown in Figure 3, identical profiles were obtained for glycans prepared by the chemical hydrazinolysis method in which there is complete release and by PNGaseF. The failure to detect any residual glycans on the IgG protein following PNGaseF treatment also suggests that release has been complete. Thus, PNGaseF proved to be a very effective method for releasing IgG N-glycans, both in terms of the overall amount and the non-selectivity of removal. This, somewhat surprising result, shows that this enzyme used under the conditions described here can be reliably used to prepare N-glycans from IgG separated from human serum. This is in agreement with previous findings by Kobata *et al.* (personal communication), who also reported non-selective release of glycans in high yield from IgG.

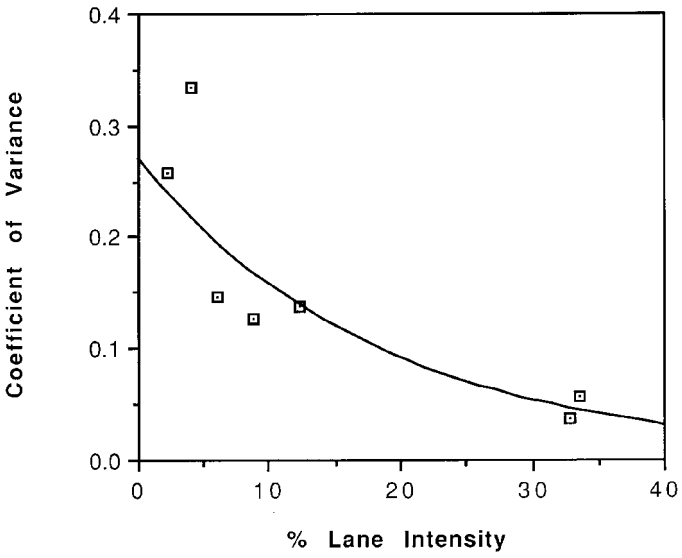

Figure 7. The coefficient of variance of IgG glycan profiling from a single sample of serum processed 11 times. The coefficient of variance was calculated for 6 of the resolved bands of differing volumes.

The small differences in molecular weights between the neutral glycans makes their resolution difficult by this technique. Although the agalactosylated, mono and di-galactosylated glycans were separated, there was no resolution of galactosylated glycans with substitutions such as fucose and N-acetyl glucosamine. These different forms could be resolved, however, by use of exoglycosidases. For example, the band corresponding to the mono-galactosylated glycans may contain structures with either fucose, bisecting N-acetyl glucosamine or both moieties. As the agalactosylated forms of these structures resolve into 3 bands, removal of the terminal galactose unit of the mono-galactosylated forms and comparison with an untreated sample would enable the determination of the ratios of glycans with these substitutions. Conversion of mono- and di- to agalactosylated glycans could be achieved by treatment of the carbohydrates with β-galactosidase.

A method of screening for IgG N-glycans would be very valuable in several working environments. For example, in

screening the glycosylation of recombinant IgG intended for therapy, comparison of different batches could be readily performed by this technique. Furthermore, in diseases such as rheumatoid arthritis where the predominance of agalactosylated IgG has been implicated in disease pathogenesis, this technique could be utilised as an indicator of disease progress and drug treatment.

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