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A general method for the complete deglycosylation of a wide variety of serum glycoproteins using peptide-*N*-glycosidase-F

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Many indirect serum studies show changes in protein glycosylation in disease, but further progress will require direct investigation of oligosaccharide composition. Current methods of deglycosylation using PNGase-F often result in incomplete removal of oligosaccharides. This is an unsatisfactory situation because only small quantities of material are often available in clinical studies, glycosylation changes may occur in only a small proportion of the molecules and quantification of the released oligosaccharides may be unreliable. The ability of PNGase-F to deglycosylate haptoglobin (Hp) under different conditions has been investigated. Oligosaccharides were completely removed from 50 μg of Hp by treatment for 24 h with PNGase-F in 50 mmol/l ammonium formate buffer, pH 8.6, in combination with sodium dodecyl sulphate, mercaptoethanol and Nonidet P40. This modified procedure was equally effective at removing oligosaccharides from other serum glycoproteins (α_1-acid glycoprotein, α_1-antitrypsin, transferrin) and fetuin, and its efficiency was independent of the polymeric structure of the molecule or the amount of glycosylation. The method has the additional advantage of using 20% less enzyme than previous methods, which substantially reduces costs.

Keywords: deglycosylation, glycoproteins, haptoglobin, PNGase-F

Introduction

Many indirect studies have reported changes in the glycosylation of serum glycoproteins in disease [1], but in most cases the precise nature of these changes has not been identified. In order to characterize these changes further, it is necessary to devise methods to release oligosaccharides from purified serum glycoproteins. A common method used for this purpose is by treatment of the glycoprotein with the enzyme peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase (peptide-*N*-glycosidase F, PNGase-F, EC 3.5.1.52). However, its effectiveness to date has been limited.

Because of its broad specificity [2], guidelines for the use of PNGase-F [3] are very general [4], and effective protocols have only been reported with either model substrates or large amounts of enzyme [5, 6]. These protocols often result in the incomplete release of oligosaccharides when adopted for routine analysis. For example, using previously published conditions [7], we were unable to deglycosylate completely small quantities of purified haptoglobin (Hp), a polymeric glycoprotein which has multiple glycosylation sites. This was carried out as part of a project studying carbohydrate changes in serum glycoproteins in alcoholic liver disease using a Dionex carbohydrate analyser [8, 9].

Complete release of oligosaccharide is important in studying glycosylation changes in disease because any abnormalities may occur in a small

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subpopulation of molecules, and only small quantities of material may be available for analysis. It was decided, therefore, to investigate the effect of various experimental conditions on the deglycosylation of Hp using PNGase-F. The improvements in the procedure that we obtained are illustrated by its effectiveness in completely deglycosylating a variety of other serum glycoproteins.

Materials and methods

Materials

Recombinant PNGase-F was obtained from Boehringer Mannheim. Human α_1-acid glycoprotein (AGP), α_1-antitrypsin (AAT), haptoglobin (Hp), transferrin (Trf) and bovine fetuin (Fet) were obtained commercially (Sigma). Details of the buffers used are shown in Table 1. The two non-ionic detergent solutions investigated in the 'modified' deglycosylation procedure were Nonidet P40 (NP40) and Triton X-100 (TX-100). These contained 2% (v/v) mercaptoethanol, 0.1 mol/l EDTA and 0.1 mol/l sodium azide. All reagents were obtained from either BDH or Sigma, and were of 'AnalaR' quality or the equivalent. Nitrocellulose membrane (Hybond-C) was obtained from Amersham. Aqueous solutions were made up with 18 MΩ cm deionized water prepared by a Milli-Q unit (Millipore) from double-distilled water.

Standard deglycosylation procedure

Using the method described by Hirani *et al.* [7], Hp (50 μg) was denatured by boiling for 5 min in 200 mmol/l sodium phosphate buffer, pH 8.6, containing 1% (w/v) sodium dodecyl sulphate (SDS) and 1% (v/v) mercaptoethanol. SDS was displaced by diluting with 200 mmol/l sodium phosphate buffer, pH 8.6, 10% (w/v) NP40 and boiling for 5 min. A 5-μl aliquot (1 BM unit, 1 BM unit = 1 mU [4]) of PNGase-F solution in 50% (v/v) glycerol was added and the solution was incubated at 37°C. This gave final reagent concentrations of Hp 0.25 mg/ml, SDS/mercaptoethanol 0.2% (w/v), NP40 1.4% and PNGase-F 5 mU/ml in a 200 μl reaction volume. A control sample of Hp containing no PNGase-F was also treated in this way. Aliquots (25 μl) were removed at 0, 24, 48 and 72 h and added to double-strength sample buffer (DSSB: 125 mmol/l Tris–HCl, pH 6.8, containing 2.7 mol/l glycerol, 1 mmol/l EDTA and 0.35 mol/l SDS). Mercaptoethanol was then added to these aliquots to give a final concentration of 5% (v/v) and the mixtures were boiled for 5 min. These samples were loaded in duplicate (1 μg and 5 μg per lane) on to a 10% (w/v) discontinuous SDS–polyacrylamide gel electrophoresis (PAGE) gel and separated for 3 h at 35 mA. After electrophoresis, the gel was cut vertically into two halves, one half (loaded with the 1-μg aliquots) being silver stained according to Thompson [10] and the other half (loaded with the 5-μg aliquots) being Western blotted onto nitrocellulose. This was carried out in Towbin transfer buffer [0.2 mol/l glycine, 25 mmol/l Tris, 20% (v/v) methanol, pH 8.6] using a BioRad Model 250 transfer system at 0.2 A for 1 h followed by 0.05 A overnight [11]. The nitrocellulose membrane was then stained for carbohydrate using the Boehringer Mannheim Glycan Detection Kit according to the manufacturer's instructions.

Table 1. The composition of the buffers used in the development of a new deglycosylation procedure. All buffers were 50 mmol/l concentration, pH 8.6, and contained 0.4% (w/v) SDS and 4 mmol/l 1,10 phenanthroline (protease inhibitor)

Buffer	Abbreviation	Composition
Ammonium formate	AF	50 ml of 100 mM $CH_2O_2NH_3$ and 1.6 ml of 100 mM NH_4OH, diluted to 100 ml
Ammonium carbonate	AC	50 mM NH_4CO_3/NH_4HCO_3
Ammonium acetate	AA	50 ml of 100 mM $CH_3CO_2NH_3$ and 13.5 ml of 100 mM CH_2CO_2H, diluted to 100 ml
Sodium phosphate	SP	50 ml of 100 mM Na_2HPO_4 and 1 ml 100 mM NaH_2PO_4, diluted to 100 ml
Clarke & Lubs solution	CL	50 ml of 100 mM KCl/H_3BO_3 and 11.8 ml of 100 mM NaOH, diluted to 100 ml
Tris–HCl	TC	50 ml of 100 mM $C_4H_{11}NO_3$ and 12.4 ml of 100 mM HCl, diluted to 100 ml
Glycine hydroxide	GO	50 ml of 100 mM $C_2H_5NO_2$ and 4 ml of 100 mM NaOH, diluted to 100 ml
Triethanolamine hydrochloride	TE	50 ml of 100 mM $C_6H_{15}NO_3HCl$ and 46 ml of 100 mM NaOH, diluted to 100 ml
Ethanolamine hydrochloride	ME	50 ml of 100 mM $C_2H_6NO_3$ and 34 ml of 100 mM HCl, diluted to 100 ml

Modified deglycosylation procedure

To develop new conditions for deglycosylating serum glycoproteins, 50 μg aliquots of a Hp solution were lyophilized and reconstituted in 50 μl of buffer or water containing various concentrations of SDS (0–1%, w/v). These were boiled for 3 min, 50 μl of a non-ionic detergent solution (0–2.5%, w/v) was added, and the solutions were boiled again for 3 min. These were then briefly centrifuged to remove liquid from the caps and left at 4°C for 10 min to cool. A 1-μl aliquot (0.2 BM units) of PNGase-F solution in 50% glycerol was added to each tube and the solutions were incubated for 24 h at 37°C. These treatments resulted in final reaction volumes of 101 μl with a PNGase-F concentration of ~2 mU/ml. Aliquots (5 μl) were removed at various time intervals (0, 5, 10, 20, 30 and 60 min and 2, 4 and 24 h) and added to an equal volume of DSSB, followed by mercaptoethanol addition and boiling as before. These were then separated on 10–15% gradient PhastGel media using a PhastSystem (Pharmacia) according to the manufacturer's instructions [12].

PhastGels were silver stained using a modification of the recommended procedure [13], which involved missing out steps 11–14 of the automated programme. After step 10, the gels were manually placed in the recommended developing solution [2.5% (w/v) sodium bicarbonate solution containing 0.01% (v/v) formaldehyde] and the pattern was developed by visual inspection. The gel was finally rinsed with deionized water and stored in 10% (v/v) glycerol solution for photography and drying. This modified approach allowed different concentrations of protein to be loaded onto separate gels without the over- or understaining problems often encountered in the automated process.

After silver staining, PhastGels were scanned using an LKB 2202 Ultrascan Laser Densitometer and 2220 Recording Integrator, and the percentage of Hp with different numbers of glycan chains was calculated for each treatment. From these data the total percentage deglycosylation could be estimated. It should be noted that for specimens with < 100% deglycosylation this was only an approximate value because of the possible effect of glycosylation on the staining procedure.

Oligosaccharide analysis

Aliquots of the digestion mixture were withdrawn after the above time intervals and microdialysed with a 1000 molecular weight cut-off (MWCO) membrane (Spectrum Medical Products) against deionized water. The dialysed material was then lyophilized to remove any remaining volatile buffer, and reconstituted in 100 μl of deionized water. The oligosaccharide sample was then separated on a CarboPac PA100 column with a 20–200 mmol/l sodium acetate gradient over 65 min in 0.1 mol/l sodium hydroxide, supplied using a Gradient Pump Module. Oligosaccharides were detected using a Pulsed Amperometric Detector-II cell and detector, with chromatographic data being collected through an Advanced Computer Interface on a 486 SX-25 PC (Wearnes) running AI-450 software. Chromatographic equipment was supplied by Dionex (UK).

Results

Figure 1 shows the silver-stained electrophoretic patterns after treating Hp with PNGase-F for different times under the 'standard' deglycosylation conditions [7]. Results are shown only for the β-chain of Hp, which has four glycosylation sites;

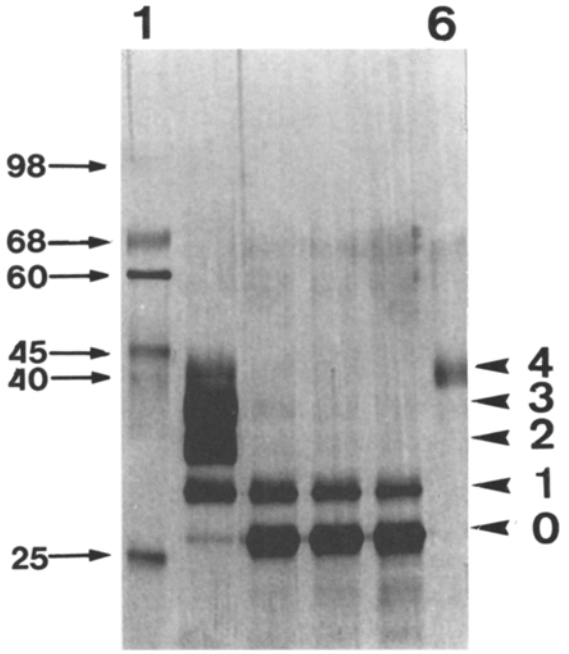

Figure 1. The effect of PNGase-F treatment for different incubation times on the electrophoretic pattern of Hp as detected by silver staining. Lanes 1 and 6 contain marker proteins and untreated Hp respectively. Lanes 2–5 contain Hp treated with PNGase-F for 0, 24, 48 and 72 h respectively. The M_r of the markers is indicated on the left-hand side of the gel. The arrowheads on the right-hand side of the figure correspond to the positions of Hp β-chains with 0–4 glycan units.

the α-chains (which have no glycosylation sites) are not shown because they run off the bottom of a 10% electrophoresis gel. With increasing time, bands of lower M_r were obtained, but it can be seen that there was incomplete removal of the oligosaccharides even after prolonged (72 h) incubation. This was further confirmed by a corresponding glycan detection blot (data not shown). This also indicated that glycan structures were absent from the Hp band of lowest M_r (~ 25 kDa), which agrees with the M_r obtained for the published amino acid sequence for human Hp [14].

Figure 2 compares the effects of: (a) various buffers, (b) the pH of the ammonium formate buffer, (c) ammonium formate buffer concentration, (d) SDS concentration, (e) NP40 concentration and (f) TX-100 concentration on the removal of carbohydrate from Hp. The approximate percentage removal of carbohydrate under the various conditions is also given in Tables 2 and 3. These experiments demonstrate that addition of

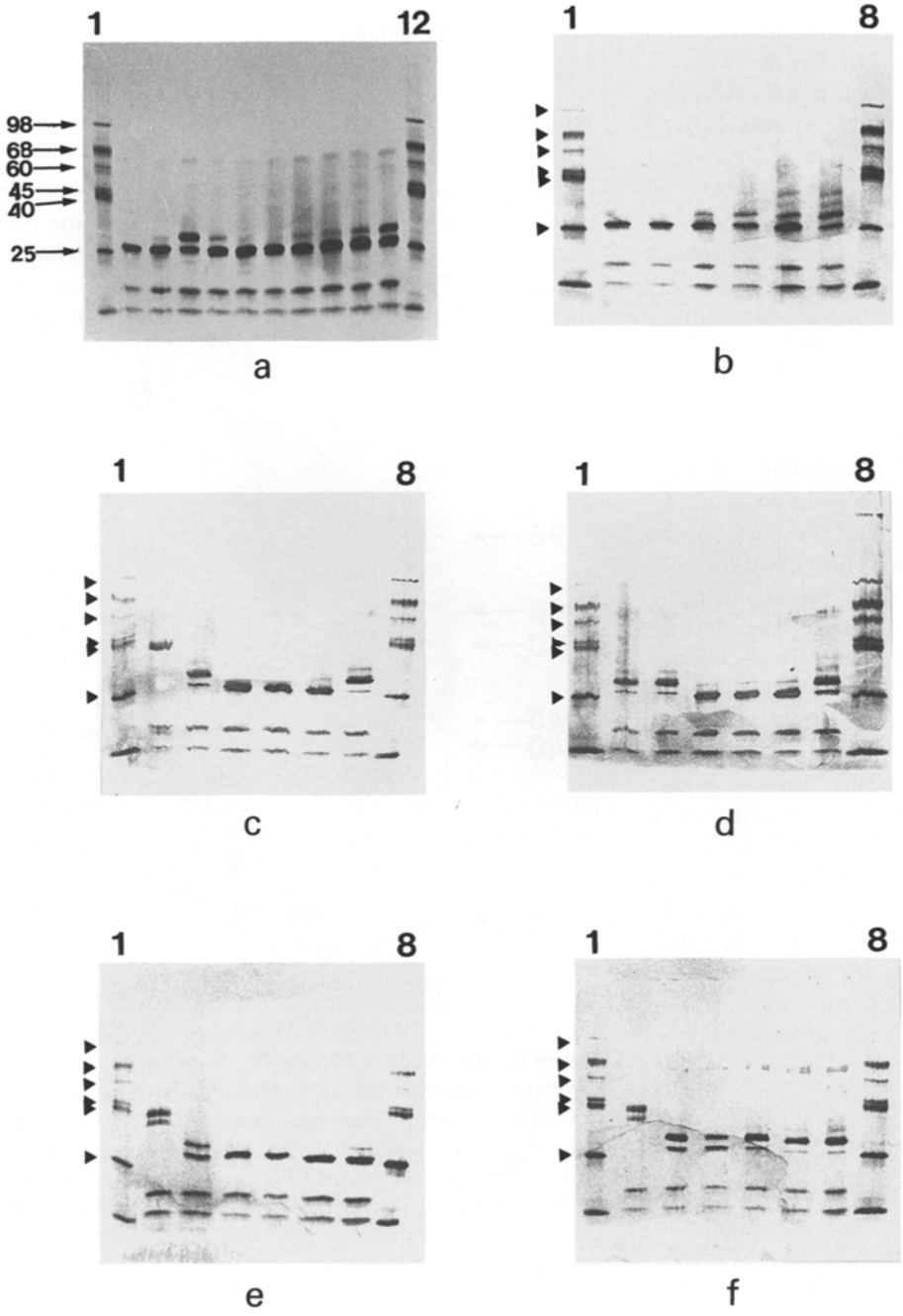

Figure 2. The effect of experimental conditions on the deglycosylation of Hp by PNGase-F. **a–f** Outside lanes = marker proteins. **a** Lanes 2–11, AF, water, AC, AA, SP, CL, TC, GO, TE and ME buffers respectively. **b** Lanes 2–7, 25, 50, 75, 100, 150 and 200 mM AF buffer respectively. **c** Lane 2, untreated Hp; lanes 3–7, pH 9.0, 8.5, 8.0, 7.5 and 7.0 respectively. **d** Lanes 2–7, 0, 0.2, 0.4, 0.6, 0.8 and 1% (w/v) SDS respectively. **e** Lanes 2–7, 0, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) NP40 respectively. **f** Lanes 2–7, 0, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) TX-100 respectively. The M_r of the markers is indicated on the left-hand side of **a**. In all other figures the position of the markers is indicated by an arrowhead on the left-hand side of the gel. The arrowheads on the right-hand side of **a** correspond to the α_1- and α_2-chains of Hp.

Table 2. Effect of buffer composition on the deglycosylation of Hp by PNGase-F

Condition investigated	Percentage of Hp with different numbers of glycan chains					Total percentage of Hp deglycosylated
	4	3	2	1	0	
(A) Buffer type						
AF	–	–	–	–	100	100
Water	–	–	–	15	85	96
AC	–	–	–	60	40	85
AA	–	–	–	49	51	88
SP	–	–	–	17	83	96
CL	–	–	–	8	92	98
TC	–	–	–	26	74	94
GO	–	–	–	22	78	95
TE	–	–	–	31	69	92
ME	–	–	–	49	51	88
(B) Ammonium formate buffer concentration (mmol/l)						
25	–	–	–	–	100	100
50	–	–	–	–	100	100
75	–	–	–	7	93	98
100	–	–	–	6	94	98
150	–	28	–	22	50	74
200	–	43	3	36	18	57
(C) Ammonium formate buffer pH						
7.0	–	–	–	62	38	85
7.5	–	–	–	19	81	95
8.0	–	–	–	11	89	97
8.5	–	–	–	–	100	100
9.0	–	–	12	81	7	74

A, B and C all used 1.2% (w/v) NP40 solution containing 2% (v/v) mercaptoethanol, 0.1 mmol/l EDTA and 0.1 mol/l sodium azide. The composition of buffers in A is given in Table 1. B used AF buffer, pH 8.6, containing 4 mmol/l 1,10-phenanthroline and 0.4% SDS. C used AF buffer 50 mmol/l containing 4 mmol/l 1,10-phenanthroline and 0.4% SDS. Percentage values were calculated from scans of silver-stained PhastGel media.

50 mmol/l ammonium formate buffer, pH 8.5, containing 0.4% SDS, followed by addition of between 1 and 1.5% NP40, is the most effective procedure to remove carbohydrate from Hp. The data also agree with previous findings [4, 15], which have demonstrated decreased activity of PNGase-F at high concentrations of buffer and SDS, and at high or low pH values.

The electrophoretic patterns for different glycoproteins (AGP, AAT, Fet, Trf, Hp) after 24 h treatment with PNGase-F under the 'modified' conditions are illustrated in Figure 3. For all the glycoproteins tested, the reduction in M_r of the protein after treatment indicated that virtually all the carbohydrate was removed [14]. The data in Figure 4 show the differences in the types of oligosaccharide removed by PNGase-F from the various glycoproteins. Figures 3 and 4 also demonstrate that complete deglycosylation of the various glycoproteins was achieved despite differences in carbohydrate content (e.g. Trf ~ 7%, AGP ~ 45% [1]) and differences in chain type respectively.

Discussion

PNGase-F is an enzyme that was discovered in *Flavobacterium meningosepticum*, which cleaves the glycosidic linkage between asparagine and *N*-acetylglucosamine in *N*-linked glycoproteins [16–18]. It is thus an asparagine amidase rather than a glycosidase as it was first classified and as some of its given names may imply. Since its discovery it has been characterized, sequenced and cloned into *Escherichia coli* [19–21], and it is commercially available. It is a valuable tool for

Table 3. Effect of the concentration of ionic and non-ionic detergent on the deglycosylation of Hp by PNGase-F

Detergent	Conc. (%, w/v)	Percentage of Hp with different numbers of glycan chains					Total percentage of Hp deglycosylated
		4	3	2	1	0	
(A) SDS concentration	0	–	–	16	67	17	75
	0.2	–	–	3	77	20	79
	0.4	–	–	–	–	100	100
	0.6	–	–	–	1	99	> 99
	0.8	–	–	–	3	97	99
	1.0	–	–	28	66	6	70
(B) NP40 concentration	0	–	49	41	10	–	40
	0.5	–	–	–	35	65	91
	1.0	–	–	–	–	100	100
	1.5	–	–	–	–	100	100
	2.0	–	–	–	13	87	97
	2.5	–	–	–	29	71	93
(C) TX-100 concentration	0	–	39	28	3	–	56
	0.5	–	–	–	53	47	87
	1.0	–	–	–	55	45	86
	1.5	–	–	–	62	38	85
	2.0	–	–	–	87	13	78
	2.5	–	–	19	67	14	74

A, B and C all used 50 mmol/l ammonium formate buffer, pH 8.6, containing 4 mmol/l 1,10-phenanthroline. A also used 1.2% (w/v) NP40 solution containing 2% (v/v) mercaptoethanol, 0.1 mol/l EDTA and 0.1 mol/l sodium azide. B and C used buffer containing 0.4% SDS. Percentage values were calculated from scans of silver-stained PhastGel medium.

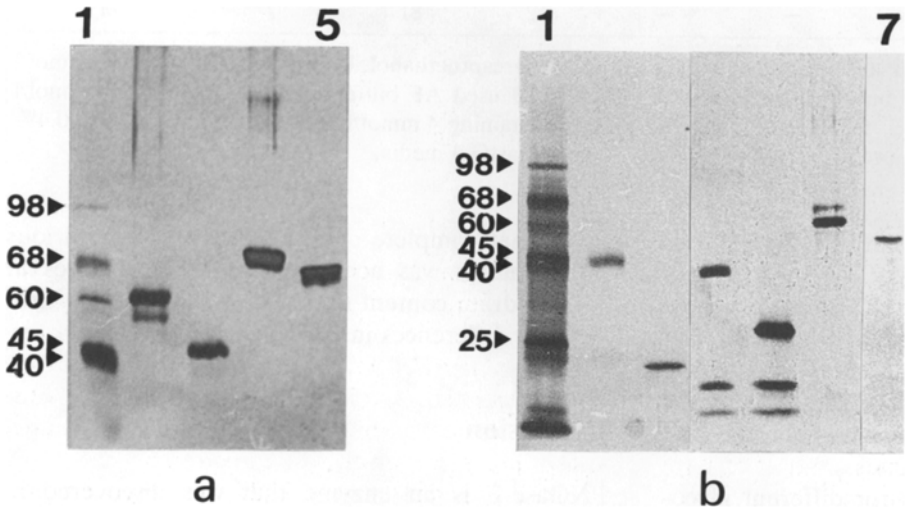

Figure 3. The silver-stained electrophoretic patterns of various serum glycoproteins deglycosylated under the conditions established for Hp. **a** **b** Lane 1, marker proteins. **a** Lanes 2 and 3 contained Fet and lanes 4 and 5 Trf treated for 0 (lanes 2 and 4) or 24 h (lanes 3 and 5) with PNGase-F. **b** Lanes 2 and 3, 4 and 5 and 6 and 7 contained AGP, Hp and AAT respectively, treated as described for Fet. The M_r of marker proteins is indicated on the left-hand side of the gels.

research on carbohydrate structure because it provides a means of preparing free oligosaccharides from virtually any glycoprotein bearing *N*-glycans. However, it has been reported to be inactive on certain structures bearing α1–3-linked core fucose [5].

In this paper we have demonstrated that previously published deglycosylation protocols, for example that described by Hirani *et al.* [7], are unable to support complete deglycosylation of a multimeric and heavily glycosylated protein such as Hp. The investigation of alternative buffer systems

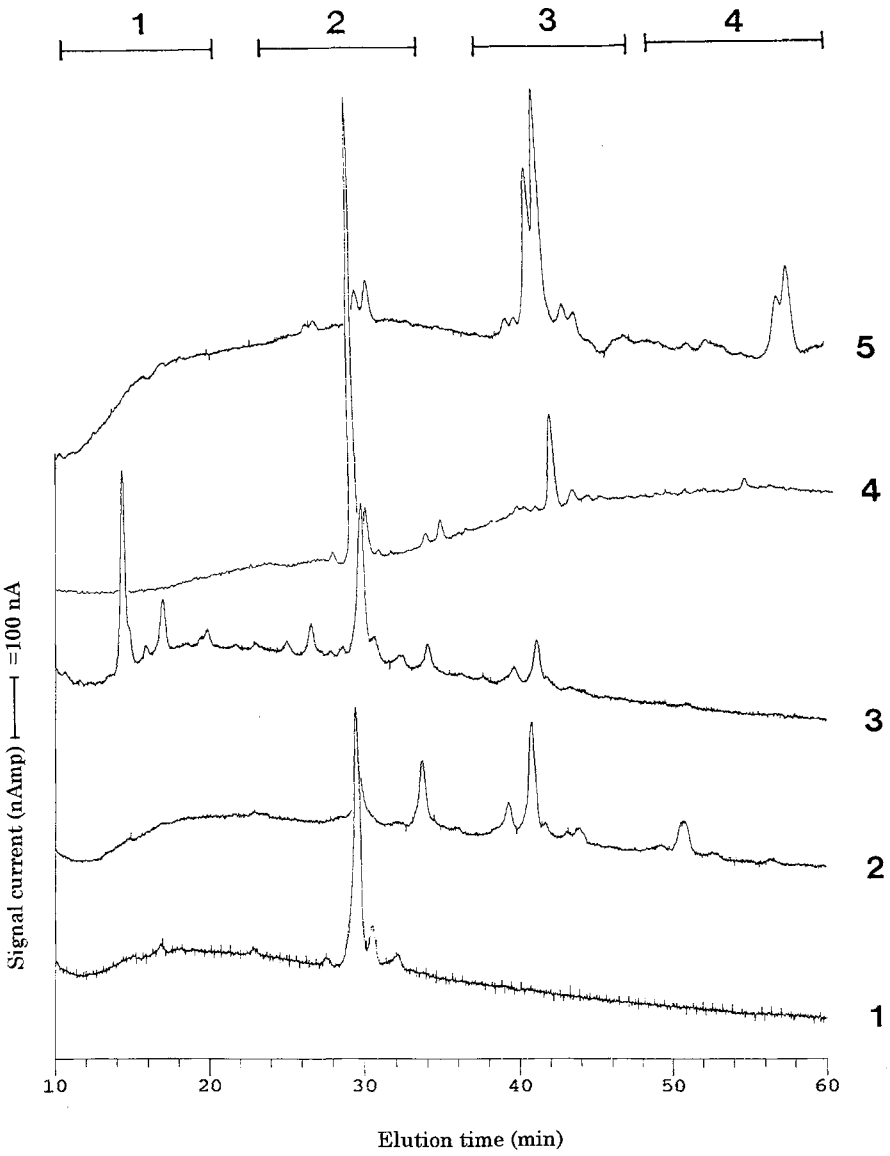

Figure 4. Sialylated oligosaccharide patterns obtained from 24 h digestion of various purified serum glycoproteins deglycosylated under the conditions established for Hp. 1, Trf; 2, AGP; 3, HP; 4, AAT; 5, Fet. Numbers at the top of the figure correspond to the number of sialic acid residues present on each group of oligosaccharides eluting within that time period.

has highlighted the usefulness of a low concentration (25–50 mM) of ammonia/ammonium formate buffer, pH 8.5, in combination with ionic/non-ionic detergents and mercaptoethanol.

The initial reasons for using an ammonia/ammonium formate buffer were twofold. Firstly, the material we use for carbohydrate analysis is purified from serum by affinity chromatography [21]. This gives 50–150 μg of purified glycoprotein in 1 ml of trifluoroacetic acid (TFA). Although most of the TFA is removed by lyophilization, a small amount remains, and is bound strongly to the protein (unpublished observations). Thus, an efficient buffer system is required to neutralize this acidity. The 50 mmol/l ammonium formate works well because it can buffer up to half a pH unit, while having a low enough concentration not to interfere with deglycosylation [15]. Secondly, the electrochemical stability of the analytical system that we use for the analysis of oligosaccharides (Dionex HPAE/PAD) is disturbed by high salt concentrations. A volatile buffer system allows the sample to be lyophilized and any residual buffer that is present after dialysis to be removed.

From our results with highly glycosylated proteins such as Fet and AGP, it seems that most glycoproteins can be deglycosylated using this protocol. However, it may not be the method of choice for some researchers who are investigating easily deglycosylated proteins such as transferrin, which has a single polypeptide chain and only two *N*-linked glycan units. In this case, incubation with just the buffer alone (without SDS) may be sufficient for complete oligosaccharide removal. We

would suggest that, upon attempting to deglycosylate a glycoprotein, three incubations are attempted – one with just buffer solution, one with buffer and non-ionic detergent solution and a final one with buffer containing SDS and a non-ionic detergent. This allows the minimal inclusion of reagents, which, in turn, facilitates a simpler procedure for the preparation of the released oligosaccharides for analysis.

It is not known why the ammonia/ammonium formate buffer system is so effective in supporting deglycosylation, although it is interesting to note that ammonia is one of the reaction products released when PNGase-F cleaves the linkage between asparagine and GlcNAc [4]. It is well known that the denaturation of a glycoprotein in SDS and mercaptoethanol, with the subsequent displacement of SDS by a non-ionic detergent, is useful in facilitating the action of PNGase-F [3], although again it is unclear exactly how this occurs.

The procedure we have developed uses small quantities of enzyme, and is very effective in deglycosylating small amounts of glycoprotein. These two advantages are extremely attractive to glycobiochemists involved with clinical research, where material is limited and early pathological changes in glycosylation may be slight. In addition, in those instances where quantification of the released oligosaccharides is relevant, confidence in complete deglycosylation is very important. It is hoped, therefore, that the resultant increase in glycan yields given by this protocol for problematic glycoproteins such as Hp will improve understanding of the changes in the glycosylation of glycoproteins in clinical conditions.

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