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Glycosidase digestion, electrophoresis and chromatographic analysis of recombinant human granulocyte colony-stimulating factor glycoforms produced in Chinese hamster ovary cells

Christi L. Clogston, Sylvia Hu, Thomas C. Boone and Hsieng S. Lu*

Amgen Inc., Amgen Center, Thousand Oaks, CA 91320 (USA)

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ABSTRACT

Recombinant human granulocyte colony stimulating factor (G-CSF) produced in Chinese hamster ovary cells is glycosylated. The carbohydrate compositional analysis indicated that G-CSF molecule contains sialic acid, galactose and galactosamine. By isolation and characterization of the purified glycopeptides obtained from cleavages by *Staphylococcal aureus* V-8 protease and cyanogen bromide, the O-linked glycosylation site was confirmed to be a Thr residue at position 133. Neuraminidase and O-glycanase digestion followed by sodium dodecyl sulfate polyacrylamide and isoelectric focusing gel electrophoreses distinguished two possible carbohydrate structures attached at Thr-133: structure A, NeuNAc-Gal-β(1,3)-GalNAc-O-Thr; and structure B, NeuNAc-Gal-β(1,3)-[NeuNAc]-GalNAc-O-Thr. Different glycoforms, undigested or after glycosidase digestion, can also be separated by ion-exchange or reversed-phase high-performance liquid chromatography. The approach described in this report provides a simple and valuable procedure to characterize glycoprotein structures containing simple carbohydrate moieties.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is one of the hematopoietic growth factors which play an important role in the stimulation, proliferation, and differentiation of hematopoietic progenitors, and are also required for functional activation of the mature cells [1,2]. G-CSF is capable of supporting neutrophil proliferation *in vivo* and *in vitro* [3–6]. The human G-CSF gene has been cloned and characterized [7,8]. Large quantities of recombinant human G-CSF (rhG-CSF) produced in genetically engineered *E. coli* have been successfully used in human clinical studies to treat neutropenic patients in a variety of clinical situations [9–13].

The natural hG-CSF isolated from tumor cell lines appears to be glycosylated [7] and has equivalent biological activity to the non-glycosylated rhG-CSF derived from *E. coli*. Due to its scarcity, characterization of the carbohydrate structure in hG-CSF isolated from a natural source has been difficult. We have expressed recombinant G-CSF in Chinese hamster ovary (CHO) cells transfected with hG-CSF cDNA to produce sufficient quantities of purified protein. In this mammalian production system, the CHO-rhG-CSF molecule, as isolated, appears to be glycosylated.

Determination of carbohydrate attachment sites and oligosaccharide structures in glycoproteins requires the strategic combination of various analytical techniques including glycosidase digestion, HPLC, isoelectric focusing (IEF)-polyacrylamide gel electrophoresis (PAGE),

* Corresponding author.

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peptide mapping, Edman degradation and amino acid analysis. Detailed analysis of oligosaccharide structures for complex glycoproteins involves the use of sophisticated instrumentation such as mass spectrometry and NMR (for reviews and refs., see refs. 14–19). We report here the analysis of the carbohydrate structures and location of an O-linked glycosylation site in CHO-rhG-CSF by sequence analysis and glycosidase digestion followed by electrophoretic analysis. The structure of oligosaccharides chemically removed from rhG-CSF has been previously determined by NMR analysis [20].

MATERIALS AND METHODS

Materials

E. coli, yeast, and CHO-cell-derived rhG-CSFs were purified by chromatographic procedures similar to those described [7]. Their purified preparations had specific activity of greater than $1 \cdot 10^8$ units/mg when assayed in a granulocyte-macrophage colony-forming unit assay [4]. Neuraminidase (sialidase, E.C. 3.2.1.18) isolated from *Vibrio cholera*, and O-glycanase (endo-α-N-acetyl-D-galactosaminidase, E.C.3.2.1.97) were purchased from Cal-Biochem and Genzyme, respectively.

Carbohydrate analysis

Acid hydrolysis of samples was carried out according to Hardy *et al.* [21]. Acid in the hydrolysate was then removed by vacuum centrifugation and then the reconstituted hydrolysate subject to composition analysis by high pH anion-exchange chromatography with pulsed amperometric detection (AE–PAD) using a Dionex BioLC system [21]. Sialic acid was quantitated spectrophotometrically according to Jourdian *et al.* [22]. Sialic acid released from neuraminidase digestion of the samples was also analyzed by AE–PAD. The separation of neutral sugars and sialic acid was separately performed on a Dionex Carbopac AS-6 (25×0.46 cm I.D.) pellicular anion-exchange column and eluted with 100 m*M* NaOH and 150 m*M* NaOAc as described previously [21,23].

Protease digestion and HPLC–peptide mapping

Reduction and S-carboxymethylation of G-CSF with dithiothreitol and iodoacetate as well as the HPLC peptide mapping were performed according to a previous procedure [24]. The alkylated derivative in 0.1 *M* ammonium bicarbonate, pH 8.3, was incubated with V-8 protease at an enzyme-to-substrate ratio of 1:30 (w/w) at 37°C for 18 h. The digestion was terminated by injection of the digest onto an HPLC column for peptide separation. Peptides were purified by reversed-phase HPLC using Vydac C-4 widepore columns (300Å: 25×0.46 cm I.D.) and an HP1090 micro liquid chromatographic system equipped with an autosampler and a photodiode array detector. Peptides were monitored at both 215 nm and 280 nm and fractions collected manually.

Amino acid sequence analysis

Polypeptides were sequenced from their amino termini by automated Edman degradation on an Applied Biosystems 477 gas-phase sequencer equipped with an on-line microbore HPLC system to detect PTH-amino acid elucidated at each degradation cycle [25]. The peptides were spotted on TFA-activated glass fiber discs which were treated with Polybrene (120 mg/ml) containing 6.7 mg/ml NaCl and preconditioned with at least two sequencer cycles.

Analytical isoelectric focusing and sodium dodecyl sulfate (SDS)-PAGE

A narrow range IEF gel (pH 5–8; thickness 0.4 mm) was prepared according to the following recipe: 6% acrylamide, 0.16% bis, 3 *M* urea, 0.05% ammonium persulfate, 6.25% servalytes (pH range 5–8). IEF was carried out at a constant power of 10 watt (voltage limit: 1500 V). The gel was prefocused for 30 minutes. Once the samples were spotted, IEF was carried out for 3.5 hours, after which the pH gradient in the gel was measured using a surface electrode (Ingold) connected to an Altex ϕ71 pH meter (Beckman). The proteins in the gel were fixed by soaking the gel in 35% methanol–10% trichloroacetic acid–3.5% sulfosalicyclic acid for 30 minutes; the proteins were stained with a solution of 0.1% Coomassie Blue R-250–35% ethanol–10%

acetic acid (staining solution was filtered through Whatman No. 1 paper before use), and the gel destained with the same solution not containing R-250.

SDS-PAGE was performed using 20 × 20 cm slabs and 15% polyacrylamide according to Laemmli [26].

Endoglycosidase digestion

Purified CHO-rhG-CSF was digested with neuraminidase according to Bhavanandan and Davidson [27] for 2 h at 37°C in a reaction buffer of 0.02 *M* Tris–maleate, 0.001 *M* $CaCl_2$, pH 6.0, and subsequently digested with O-glycanase for 6 h at 37°C [28].

Reversed-phase and cation-exchange HPLC

Reversed-phase HPLC separation was performed using procedures described previously [29]. The HPLC analysis was carried out on a C_4 reversed-phase column (25 × 0.46 cm I.D.; Vydac wide-pore) employing trifluoroacetic acid (TFA)–acetonitrile gradient elution at a flow-rate of 0.8 ml/min at room temperature.

Separation of CHO-rhG-CSF glycoforms was also performed using a TSK SP-5PW cation-exchange column (7.5 × 0.75 cm I.D., Tosoha, Japan). The column was developed with a 85-min gradient of 0 to 75 m*M* sodium sulfate in 20 m*M* sodium acetate, pH 5.4. The flow-rate was 0.7 ml/min and the column effluent was monitored at 230 nm.

RESULTS AND DISCUSSION

Carbohydrate composition

The following compositional analysis confirms that hG-SF expressed in CHO cells is glycosylated. After a desalting step, purified CHO-rhG-CSF was subject to partial acid hydrolysis and carbohydrate analysis. Data shown in Table I is the carbohydrate composition expressed in molar ratio. The results indicate that CHO-rhG-CSF contains a simple carbohydrate composition, *i.e.*, 1–2 mol of sialic acid residues and one residue each of galactose and galactosamine. The content of sialic acid quantitated by the spectrophotometric method is consistent with that determined by HPAE–PAD analysis of the neuraminidase-digested sample. The carbohydrate composition shown above suggests that CHO-rhG-CSF may contain simple Thr/Ser-linked carbohydrate moieties.

TABLE I

CARBOHYDRATE COMPOSITION OF RECOMBINANT HUMAN G-CSF EXPRESSED IN CHO CELLS

Sugar[a]	mol/mol[b]
Gal	1.19
GalNAc	1.00
NeuNAc	1.42[c]
	1.67[d]

[a] Neutral sugars were analyzed by AE–PAD after acid hydrolysis.
[b] Numbers are expressed as mol carbohydrate per mol G-CSF.
[c] Analyzed by spectrophotometric assay.
[d] Analyzed by AE–PAD for neuraminidase-digested sample.

Assignment of a single O-linked sugar attachment site

The location of potential glycosylation sites was assigned by isolation and structural characterization of glycopeptides. In peptide map analysis, purified *E. coli* and CHO-derived G-CSFs were reduced with dithiothreitol to break the disulfide bonds and subsequently alkylated with iodoacetate. These S-carboxymethylated derivatives were digested with *S. aureus* V-8 protease followed by reverse-phase HPLC analyses to isolate peptides. As shown in Fig. 1, the G-CSF digests produced comparable peptide maps between the two samples with only two notable exceptions: (1) the major peptide peak at 49 min in CHO-derived G-CSF had shifted to 50 min in *E. coli* G-CSFs map. These peptides were verified to be the N-terminal peptides of G-CSF. The increase in retention time of *E. coli* G-CSF N-terminal peptide is caused by the additional methionine present in the molecule; and (2) another peptide found at 63 min in the *E. coli*-derived G-CSF map had shifted to earlier retention at 60.5 min in the CHO-rhG-CSF map and elutes as a broader peak. These peptides were confirmed to be the C-terminal peptides of G-CSF. The earlier elution and broadness of the peak shape for the CHO-rhG-CSF C-terminal

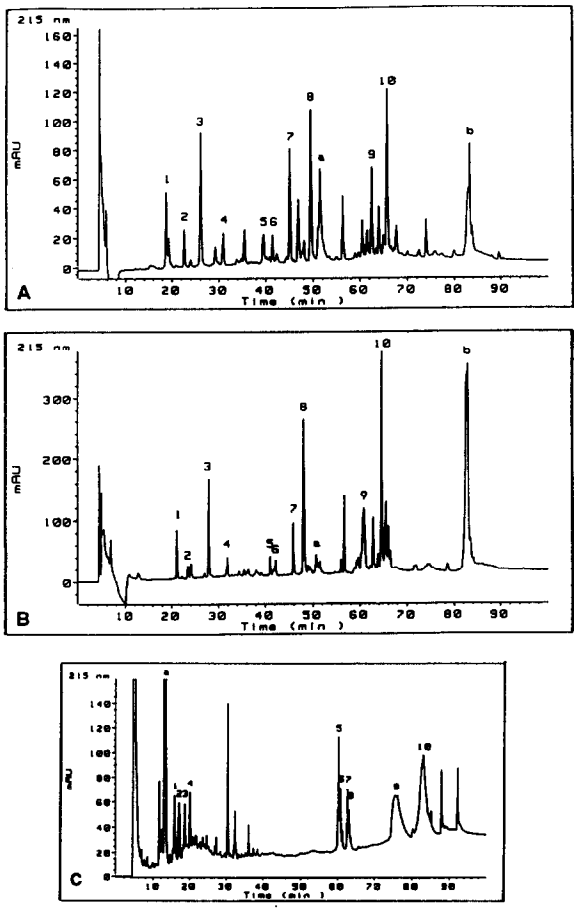

Fig. 1. HPLC peptide maps of different G-CSF preparations derived from digestions using *S. aureus* V-8 protease. (A) Peptide map of carboxymethylated rhG-CSF produced in *E. coli* (75 μg). (B) Peptide map of carboxymethylated CHO-rhG-CSF (75 μg). (C) Peptide map of cyanogen bromide peptides for CHO-rhG-CSF. Solvent A = 0.1% TFA; solvent B = TFA–H_2O–CH_3CN (0.1:9.9:90). The column was initially equilibrated at 97% A–3% B at a flow-rate of 0.7 ml/min. The peptides were separated by a linear gradient from 3% B to 35% B over 10 min followed by 80% B isocratic elution for 20 min. Peptides were numbered arbitrarily according to their elution orders. Peak a is an artifact peak derived from carboxymethylation and peak b is the undigested core protein. Several unnumbered peptides in chromatograms A and B represent those derived from non-specific cleavages.

peptide indicates that this peptide contains heterogeneous carbohydrate moieties.

Amino acid sequence of the C-terminal peptide (t_R 60.5 min) obtained from peptide mapping of the carboxymethylated CHO-rhG-CSF derivative begins with Leu-124 and contains five potential O-linked glycosylation sites, *i.e.*, Thr-133, Ser-142, Ser-155, Ser-159 and Ser-164. Among the first four potential glycosylation sites, only Thr-133 yielded no signal during N-terminal sequence analysis, making it a potential site of glycosylation, however, Ser-164 has yet to be eliminated as a possible site. Table II summarizes the quantitative recovery of PTH-amino acids obtained from automated gas phase Edman degradation of peptide.

CHO-rhG-CSF was further digested with cyanogen bromide to generate larger fragments cleaved at methionine. After separating by HPLC (Fig. 1C), a peak at 60 min was recovered and found to be the C-terminal peptide beginning with Pro-138. This peptide was sequenced through Tyr-165. Recovery of PTH-amino acid can be clearly found at Ser-142, Ser-155, Ser-159 and Ser-164, thus eliminating these serines as potential glycosylation sites. Another peptide with a retention time of 20 min was found to be the Ala-127 to Met-137 peptide. Sequence analysis indicated that Thr-133 is the only amino acid which cannot be detected (Table II). This result corroborates the previous analytical data obtained from a V-8 protease-generated C-terminal peptide described above. These results conclude that the hydroxyl side chain of Thr-133 is the only amino acid linked to carbohydrate moieties in CHO-G-CSF.

Glycosidase digestion and electrophoretic analysis

By taking advantage of the selective specificity of endoglycosidases in hydrolyzing glycosidic bonds of oligosaccharides in glycoproteins, we further studied the carbohydrate structure of CHO-rhG-CSF by neuraminidase and O-glycanase digestion. Neuraminidase catalyzes the hydrolysis of α(2,3)-, α(2,6)-, or α(2,8)-linked sialic acids on oligosaccharides and glycoproteins while O-glycanase catalyzes the hydrolysis of the Gal-β(1,3)-GalNAc core disaccharides attached to serine or threonine residues of glycoproteins or glycopeptides [27,28]. CHO-rhG-CSF was first treated with neuraminidase for 2 hours. A portion of the digest was used to determine concentration of the released sialic acid by HPAE–PAD

TABLE II

SEQUENCE ANALYSIS OF TWO CHO-rhG-CSF GLYCOPEPTIDES AND A C-TERMINAL PEPTIDE

Sequence position	Peptides					
	S-9[a]		CNBr-5[b]		CNBr-4[b]	
	Amino acid	Recovery (pmol)	Amino acid	Recovery (pmol)	Amino acid	Recovery (pmol)
124	Leu	1000				
125	Gly	771				
126	Met	348				
127	Ala	348			Ala	157
128	Pro	171			Pro	62
129	Ala	175			Ala	81
130	Leu	284			Leu	109
131	Gln	128			Gln	52
132	Pro	101			Pro	31
133	( )	ND[c]			( )	ND[c]
134	Gln	66			Gln	26
135	Gly	105			Gly	30
136	Ala	61			Ala	4
137	Met	34				
138	Pro	39	Pro	344		
139	Ala	26	Ala	302		
140	Phe	28	Phe	333		
141	Ala	17	Ala	266		
142	Ser	14	Ser	93		
143	Ala	8	Ala	111		
144	Phe	14	Phe	240		
145	Gln	18	Gln	64		
146	Arg	8	Ala	98		
147	Arg	3	Arg	33		
148	Ala	8	Ala	98		
149	Gly	14	Gly	161		
150	Gly	3	Gly	33		
151	Val	3	Val	107		
152	Leu	4	Leu	142		
153	Val	1	Val	104		
154	Ala	4	Ala	113		
155	Ser	3	Ser	25		
156	His	2	His	4		
157	Leu	4	Leu	67		
158	Gln	3	Gln	28		
159	Ser	1	Ser	11		
160	Phe	1	Phe	53		
161			Leu	27		
162			Glu	8		
163			Val	26		
164			Ser	8		
165			Tyr	9		

[a] Isolated from peptide map from *S. aureus* V-8 protease digest of carboxymethylated CHO-rhG-CSF (see Fig. 1B).
[b] Isolated from peptide map from CNBr cleavage of carboxymethylated CHO-rhG-CSF (see Fig. 1C).
[c] Not detected.

(see Methods). The resulting asialo molecule in another portion of the digest was subsequently digested with O-glycanase for 8 hours to cleave any asialo O-linked sugar. As indicated in Fig. 2, by SDS-PAGE, the neuraminidase-treated G-CSF has M_r 19 200 as opposed to M_r 19 600–20 000 for the untreated sample. Subsequent treatment with O-glycanase further reduced the mass of G-CSF to approximately 18 800. Since neuraminidase and O-glycanase are highly specific glycosidases, the molecular mass reduction of CHO-rhG-CSF by these two hydrolases suggests a possible structure for the carbohydrate component, *i.e.*, NeuNAc-Gal-β(1,3)-GalNAc-O-Thr-133.

The endoglycosidase-treated CHO-rG-CSF was also analyzed by narrow range IEF (Fig. 3). Digestion of samples with neuraminidase results in the disappearance of the diminution for p*I* 5.5 band, a partial decrease for p*I* 5.8 form, and the appearance of a new band at p*I* 6.15. This experiment suggests that the p*I* 5.8 G-CSF band contains one sialic acid and the p*I* 5.5 band two sialic acids, as the removal of sialic acid had generated asialo protein with identical net charges, migrating at a p*I* around 6.15. IEF analysis also shows that the enzyme hydrolyzes the p*I* 5.5 form containing two sialic acids more efficiently than the p*I* 5.8 form containing a single sialic acid. Further treatment of desialylated protein with O-glycanase results in no further change in p*I*, indicating that O-glycanase only removes the expected neutral sugars.

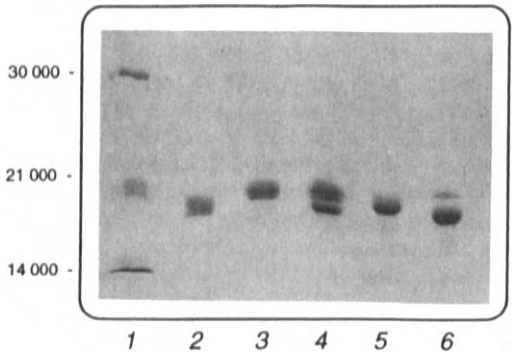

Fig. 2. SDS-polyacrylamide gel electrophoresis. Lanes: 1 = standard protein markers; 2 = rhG-CSF produced in *E. coli*; 3 = CHO-rhG-CSF; 4 = CHO-rhG-CSF and *E. coli*-produced rhG-CSF; 5 = CHO-rhG-CSF treated with neuraminidase; and 6 = CHO-rhG-CSF treated with neuraminidase and O-glycanase (5 μg load on each gel lane).

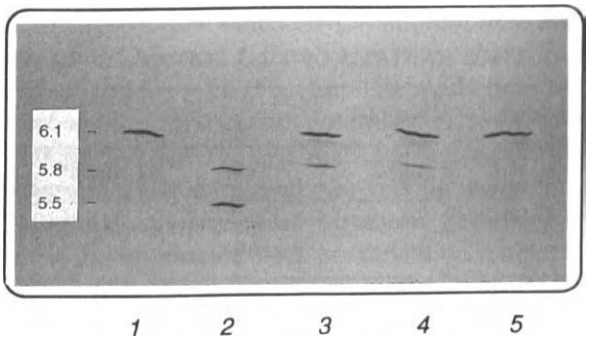

Fig. 3. Narrow-range isoelectric focusing gel electrophoresis. Lanes: 1 and 5 = *E. coli*-produced rhG-CSF; 2 = CHO-rhG-CSF; 3 = CHO-rhG-CSF treated with neuraminidase; and 4 = CHO-rhG-CSF treated with neuraminidase and O-glycanase (2.5 μg loaded on each lane).

Changes in both molecular mass and p*I* for CHO-rhG-CSF after neuraminidase and O-glycanase treatment suggests two possible carbohydrate structures: structure A, NeuNAc-Gal-β(1,3)-GalNAc-O-Thr-133; and structure B, NeuNAc-Gal-β(1,3)-[NeuNAc]-GalNAc-O-Thr-133. The linkage between NeuAc and Gal could be α(2,3) or α(2,6). The α(2,6) linkage is the only possibility connecting the NeuAc to GalNAc in structure B. Under narrow range IEF, CHO-rhG-CSF containing structure A would migrate at p*I* 5.8 and structure B at p*I* 5.5, while non-glycosylated *E. coli*-derived and yeast-derived rhG-CSFs as well as asialo CHO-rhG-CSF containing neutral sugar migrates at p*I* 6.1. This data is consistent with that derived from NMR analysis described previously [20].

Asialo CHO-rhG-CSF and the completely deglycosylated molecule can be clearly differentiated from untreated samples by IEF and SDS-PAGE. These analyses led us to conclude that Thr-133 in CHO-rhG-CSF is fully attached with carbohydrate moieties; the purified material does not contain a non-glycosylated form. Asialo CHO-G-CSF, which still contains neutral sugar, migrates at a p*I* identical to non-glycosylated G-CSF by IEF but is distinguishable by SDS-PAGE. This is supported by data obtained from reversed-phase and cation-exchange HPLC, described below.

Characterization of rhG-CSF glycoforms by chromatographic separation

Shown in Fig. 4A is the reversed-phase HPLC separation of CHO-rhG-CSF, asialo CHO-rhG-CSF obtained from neuraminidase treatment, and deglycosylated CHO-rhG-CSF after O-glycanase treatment. The results show that removal of carbohydrates increases the retention of the deglycosylated molecule in a reversed-phase column, suggesting an increase in hydrophobicity due to deglycosylation. However, CHO-rhG-CSF species varying in content of sialic acids are not separable in reversed-phase HPLC (Fig. 4A, chromatogram 1). Instead, these species can be well separated by ion-exchange HPLC shown in Fig. 4B. Ion-exchange HPLC completely resolves CHO-rhG-CSF into two major species, in approximately equimolar ratio. Peak I was identified as the glycosylated G-CSF containing two sialic acids and peak II as the glycoform with only one sialic acid (data not shown). Both asialo CHO-rhG-CSF and non-glycosylated hG-CSF (derived from yeast expression) coelute, but are also separable from peak II. They elute 4–5 min later than peak II in cation-exchange HPLC (data not shown).

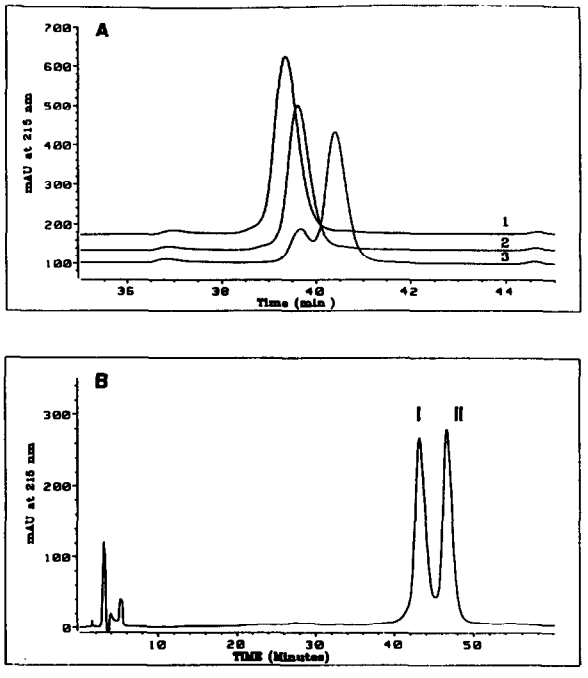

Fig. 4. (A) Reversed-phase HPLC of G-CSF species. Chromatograms 1–3 (25 μg each): CHO-rhG-CSF; asialo CHO-rhG-CSF treated with neuraminidase; and CHO-rhG-CSF treated with neuraminidase and O-glycanase, respectively. (B) Cation-exchange HPLC of CHO-rhG-CSF (50 μg injected).

In contrast to the above data, Nomura *et al.* [30] had reported that human G-CSF produced by a tumor cell line exhibits three distinct p*I* forms (5.5, 5.8 and 6.1). The p*I* 5.5 and 5.8 subforms match the sialylated subforms found in CHO-rhG-CSF, while the 6.1 subform, not detected in CHO-rhG-CSF, may be the asialo or non-glycosylated G-CSF.

CONCLUSION AND DISCUSSION

Experimental approaches using glycosidase digestion in combination with electrophoretic analysis provide useful procedures to study simple carbohydrate structure in glycoproteins. SDS-PAGE can distinguish molecular mass reduction of glycoprotein after deglycosylation, while IEF electrophoresis can identify difference in a single net charge upon removal of sialic acid during neuraminidase digestion. The experimental results yield simple as well as interpretable data and provide further insight on the understanding of carbohydrate structure for small molecular mass glycoproteins. Elucidation of detailed carbohydrate structure is usually not attainable without applying sophisticated techniques such as NMR spectroscopy. However, the NMR analysis requires utilization of the highly expensive equipment and time-consuming preparation of purified oligosaccharides in large quantities. The procedure described here offers an inexpensive and reliable alternative to laboratories which do not have access to the costly equipment. However, this approach may not be applicable to glycoproteins with complex N- and O-linked carbohydrate structures.

The presence of carbohydrate on human G-CSF appears to have little effect on G-CSFs ability to promote the proliferation and differentiation of the granulocyte progenitor lineages *in vitro* or *in vivo* [3,4,7]. One explanation may be that the threonine-linked sugar structure in recombinant human G-CSF is very small and may

not affect the rate of clearance of the molecule *in vivo* so as to alter the biological activity. Moreover, no detectable difference in their stability was observed between glycosylated G-CSF and non-glycosylated G-CSF (our unpublished data).

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