Structural Characterization of Recombinant Human Erythropoietins by Fluorophore-Assisted Carbohydrate Electrophoresis

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Routine analysis of the carbohydrate moieties of a glycoprotein is critical for ensuring the consistent quality of biopharmaceutical products. Fluorophore-assisted carbohydrate electrophoresis (FACE) is a recently introduced method for the separation, by polyacrylamide gel electrophoresis, of oligosaccharides labeled with 8-amino-naphthalene-1,3,6-trisulphonic acid (ANTS).

In this study, we have evaluated the applicability of the FACE method to analysis of the carbohydrate moieties of three different recombinant human erythropoietins (rHuEPOs), two Chinese hamster ovary (CHO) cell-derived rHuEPOs, and one baby hamster kidney (BHK) cell-derived rHuEPO. The *N*-linked oligosaccharides released from the rHuEPOs were labeled with ANTS. Enzymic sequence analysis of the *N*-linked oligosaccharides was performed by the FACE method. The results showed that the FACE method was useful for the analysis of sialo-, asialo-, and agalacto-oligosaccharides in the same gel. Its usefulness for rapidly and reliably revealing the oligosaccharide profiles of given glycoproteins, and its reproducibility were also confirmed in this study. In conclusion, the method can be used for evaluating the quality consistency of recombinant glycoprotein products in terms of the carbohydrate moiety.

Key words recombinant human erythropoietin; FACE; *N*-linked oligosaccharide; sequencing; exoglycosidase digestion

Many kinds of cytokines, including erythropoietin (EPO), granulocyte-macrophage-stimulating factor, macrophage colony-stimulating factor and some interferons, are glycoproteins. The important roles of oligosaccharide chains in the biological activities of these cytokines have been reported.^{2–4)} The primary structure of the polypeptide chain sequence is strictly dependent on the DNA sequence, but the oligosaccharide chain sequence is not directly determined by the DNA sequence. The biosynthesis of oligosaccharide chains is influenced not only by the host-cells, but also by the structure of the polypeptide to which an oligosaccharide chain is added.^{5,6)} Therefore, it is essential to ensure the consistency of glycosylation during the repeated production of a recombinant glycoprotein. For recombinant glycoproteins, it is therefore necessary to determine the extent of glycosylation by a method that is both practical and sufficient for each stage of product development and production, and to bear clearly in mind why the analysis is being performed.

Recombinant human erythropoietin (rHuEPO) is a glycoprotein with one *O*-linked and three *N*-linked oligosaccharide chains, comprising about 40% of its molecular mass.^{7–9)} The relationship between the oligosaccharide structure and its functions in rHuEPO has been studied in detail in literature including in our papers.^{10–12)}

A significant recent development in carbohydrate analysis has been an application of various kinds of high-performance liquid chromatography (HPLC) on two-dimensional mapping techniques and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).^{3,4)} Fluorophore-assisted carbohydrate electrophoresis (FACE) was developed by Jackson.^{13–15)} This involves a technique in which a negatively charged fluorescent tag, 8-amino-naphthalene-1,3,6-trisulphonic acid (ANTS), is coupled to the free reducing termini of oligosaccharides by re-

ductive amination with high efficiency. The FACE method is a recently introduced technique which can be used to analyze the structure of oligosaccharides by means of separation on polyacrylamide gel electrophoresis (PAGE).¹⁶⁾

In this study, we applied this method to the analysis of *N*-linked oligosaccharides derived from three different rHuEPOs, and examined its usefulness for the quality control of glycoprotein products.

MATERIALS AND METHODS

Materials The two rHuEPOs produced by Chinese hamster ovary (CHO) cells were kind gifts from Chugai Pharmaceutical Co., Ltd. and Kirin Brewery, Japan, and the rHuEPO from baby hamster kidney (BHK) cells was kindly donated by Snow Brand Milk Products, Japan. ANTS disodium salt, was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Peptide-N⁴-(*N*-acetyl- β -glucosaminy) asparagine amidase (PNGase F), β -galactosidase (GALase, *Streptococcus* 6646 K and jack bean) were obtained from Seikagaku Corporation (Tokyo, Japan). Neuraminidase (NANase, *Arthrobacter ureafaciens*) and Nonidet P-40 were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). β -*N*-Acetylglucosaminidase (HEXase, HEXase III from *E. coli*), ANTS-labelled glucose polymers 1–20 (oligo ladder), FACE *N*-linked-oligosaccharide profiling gels and buffer, and *N*-linked Oligosaccharide Sequencing Kits were obtained from Glyko, Inc., Novato, CA, U.S.A. Native *N*-linked oligosaccharides, including asialo-galactosylated tetraantennary, asialo-agalacto-tetraantennary, asialo-galactosylated triantennary, asialo-agalacto-triantennary, asialo-galactosylated biantennary and asialo-agalacto-biantennary ones, and the conserved trimannosyl core substituted with fucose were obtained from Oxford Glyco Systems, Inc. (Rosedale, NY,

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U.S.A.). Sodium cyanoborohydride and sodium dodecyl sulfate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Release and Labeling of *N*-Linked Oligosaccharides from rHuEPO One hundred μg of rHuEPO was dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1% SDS and 0.25 M 2-mercaptoethanol, followed by heating at 95 °C for 5 min. After cooling to room temperature, Nonidet P-40 was added to a final concentration of 0.75%, followed by digestion with 2 mU of PNGase F for 2 h at 37 °C. The released oligosaccharides were recovered by precipitation of the protein with 3 vol. of cold ethanol and drying the supernatant in a centrifugal concentrator (TAIYO VC-36, Japan), and then stored at -20 °C.

Fluorophore Labeling Reaction The released oligosaccharides and authentic native *N*-linked oligosaccharides were labeled by adding 5 μl of 0.15 M ANTS in 15% acetic acid and 5 μl of 1.0 M sodium cyanoborohydride in DMSO, followed by incubation at 37 °C for 16 h and drying in a centrifugal concentrator.¹⁶⁾

Sequential Exoglycosidase Digestion of *N*-Linked Oligosaccharides Derived from rHuEPO Dried oligosaccharides were dissolved in 20 μl of 100 mM citrate buffer (pH 3.5), then 2 μl of the solution was transferred into each of 5 tubes. Glycosidase digestion was performed sequentially with 10 mU of NANase in 10 mM phosphate buffer (pH 7.3) for 12 h, 5 mU of GALase (*Streptococcus*) in 10 mM phosphate buffer (pH 7.2), 20 mU of GALase (jack bean) in 100 mM citrate buffer (pH 5.0), and 84 mU of HEXase in 20 mM Tris-HCl containing 25 mM NaCl (pH 7.2), for 16 h at 37 °C.

Electrophoresis The labeled oligosaccharides were separated by PAGE on an *N*-linked oligosaccharide profiling gel (Glyko, Inc.) containing 21% polyacrylamide at a current of 15–20 mA and 4 °C for 1–1.5 h.

Fig. 1. Profiling of Sialo- and Asialo-*N*-linked Oligosaccharides Derived from 3 rHuEPOs

Lane 1 contained oligoladder standards consisting of ANTS-labeled glucose polymers 1–20 containing 50 pmol of maltotetraose (G4); lanes 2, 3 and 4 contained *N*-linked oligosaccharides from samples A, B, and C without enzymic treatment, respectively; lanes 5, 6 and 7 contained *N*-linked oligosaccharides from samples A, B and C with NANase treatment, respectively.

Table 1. Relative Amounts of Sialo-*N*-linked Oligosaccharide in Each EPO Sample

Sample	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7
Sample A	4.4 $\pm$ 1.9	3.4 $\pm$ 1.5	15.5 $\pm$ 3.5	13.5 $\pm$ 3.7	37.6 $\pm$ 3.1	11.8 $\pm$ 0.8	13.7 $\pm$ 4.2
Sample B	6.8 $\pm$ 1.5	4.5 $\pm$ 1.5	20.2 $\pm$ 3.1	9.2 $\pm$ 3.9	38.0 $\pm$ 3.4	12.2 $\pm$ 1.6	9.0 $\pm$ 3.3
Sample C	6.7 $\pm$ 1.0	3.6 $\pm$ 1.3	20.8 $\pm$ 1.9	9.2 $\pm$ 2.9	41.4 $\pm$ 3.3	11.3 $\pm$ 3.0	7.0 $\pm$ 1.7

Values are mean $\pm$ S.D. of 10 experiments.

FACE Imaging Analysis of the Gels The gels were imaged using a FACE Imaging System. A FACE workstation with Imager and FACE imaging software version 2.30 from Glyko, Inc. were used throughout these studies.

RESULTS

FACE Profiling of 3 rHuEPOs Oligosaccharides labeled with ANTS obtained from 6–8 μg of EPO were subjected to PAGE. Lane 1 in Fig. 1 contained the OLIGO Ladder Standard, which is a mixture of glucose polymers. Undigested oligosaccharide profiles of three rHu-EPOs are shown in lanes 2–4. Almost similar patterns, including 7 bands, were observed for the 3 samples. From top to bottom, the bands were numbered 1–7. The main band was band 5. After digestion with 10 mU of NANase (*Arthrobacter ureafaciens*), all the bands shifted upward, as shown in lanes 5–7. This indicates the presence of sialic acid in most of the oligosaccharides. Based on the results of densitometric analysis, the relative amounts of sialo- and asialo-oligosaccharide in each rHuEPO sample were estimated as shown in Tables 1 and 2. The averaged data and their S.D. values were obtained for ten experiments including the release and labeling of *N*-linked oligosaccharides from each EPO product. The results indicate that the FACE method with charge-coupled device (CCD) imaging was reproducible. Each band could be detected with as little as 1 pmol of an oligosaccharide.

While the pattern of asialo-oligosaccharides of sample A from CHO cells exhibited a similar pattern to that of sample B from other CHO cells (Table 2), the pattern of sialo-oligosaccharides of sample A was slightly different from that of sample B (Table 1). The patterns of sialo- and asialo-oligosaccharide of sample C were different from those of sample A (Tables 1 and 2). Next, the relative amounts of asialo-oligosaccharides in samples A, B and C were calculated from the published data^{7–9)} in terms of bi-, tri- and tetra-antennary structure, as well as the number of lactosamine repeat units, and these are presented in Fig. 2. The distribution pattern of asialo-oligosaccharides in each sample obtained in this retrieval survey showed good correspondence with the pattern of asialo-oligosaccharides in the present study. From their relative quantities and mobilities, bands

Table 2. Relative Amounts of Asialo-*N*-linked Oligosaccharide in Each EPO Sample Treated with NANase

Sample	Band 1	Band 2	Band 3	Band 4	Band 5
Sample A	4.9 $\pm$ 1.9	21.0 $\pm$ 3.4	49.9 $\pm$ 2.9	12.4 $\pm$ 3.2	11.8 $\pm$ 5.4
Sample B	6.1 $\pm$ 1.5	22.0 $\pm$ 3.7	47.6 $\pm$ 4.5	13.6 $\pm$ 4.8	10.7 $\pm$ 3.8
Sample C	5.3 $\pm$ 0.4	14.7 $\pm$ 2.2	41.1 $\pm$ 4.9	20.1 $\pm$ 4.7	18.6 $\pm$ 2.9

Values are mean $\pm$ S.D. of 10 experiments.

Fig. 2. Distribution Patterns of Asialo-N-linked Oligosaccharides, Cited from References

Fig. 3. Enzymic Sequencing of 2 different Types of rHuEPOs

Lane 1, oligoladder standards; lanes 2, 3, 4 and 5, N-linked oligosaccharides from sample A, treated without an enzyme and with NANase, NANase+GALase, and NANase+GALase+HEXase, respectively; lane 6, 7 and 8, N-linked oligosaccharides from sample B, treated with NANase, NANase+GALase, and NANase+GALase+HEXase, respectively.

1, 2, 3, 4 and 5 in Table 2 and Fig. 1 could be assigned as biantennary, triantennary, tetraantennary, tetraantennary with one N-acetylglucosamine unit, and tetraantennary with two or three N-acetylglucosamine units, respectively.

FACE Sequencing of N-Linked Oligosaccharides from rHuEPOs Since the bands of asialo-oligosaccharides could not be digested fully with the purchased N-linked Oligosaccharide Sequencing Kits (data not shown), we performed a preliminary study using various kinds of GALase and HEXase. The optimal digestion conditions, under which they could be digested fully, were 5 mU of GALase (*Streptococcus*) plus 20 mU of GALase (jack bean), and 84 mU of HEXase were used as described under Materials and Methods.

Figure 3 shows typical patterns obtained on the enzymatic sequencing of samples A and B. Lane 2 contained oligosaccharides from sample A. Lanes 3 and 6 contained NANase-treated oligosaccharides from samples A and B. Lanes 4 and

7 contained NANase- and GALase-treated oligosaccharides, and lanes 5 and 8 NANase-, GALase- and HEXase- treated oligosaccharides from samples A and B, respectively. All the oligosaccharide bands for untreated samples clearly shifted upward on NANase treatment, and then shifted downward on additional GALase and HEXase treatments.

The positions of the oligosaccharides in each lane are presented as the "degree of polymerization" (DP) using the ladder in lane 1 as a standard oligosaccharide (G4 has a DP=4.0) as defined by Starr *et al.*¹⁶⁾ The DP of the main band for sample A was estimated to be 7.7 in lane 2, 12.3 in lane 3, 8.2 in lane 4, and 4.9 in lane 5 (Fig. 3). Digestion of the original material with NANase plus GALase (Fig. 3, lane 4) caused a downward shift of all five bands relative to their positions in lane 3. The change in DP between these adjacent lanes suggests a loss of 4 Gal residues. Further digestion with these 2 enzymes plus HEXase resulted in only one band (Fig. 3, lane 5), which co-migrated with the conserved trimannosyl core substituted with fucose (Fig. 4, lane 7).

The DPs of seven authentic ANTS standards were compared with those of the enzymic sequencing mixture for sample A (Fig. 4). A standard mixture of authentic ANTS-labeled oligosaccharides containing galactosylated tetraantennary, galactosylated triantennary and galactosylated biantennary sugar chains was applied to lane 4, and the DP were determined to be 11.8, 10.2 and 8.7, respectively. The DPs of the ANTS-labeled agalacto-tetraantennary, agalacto-triantennary and agalacto-biantennary sugar chains in lane 6 were 7.7, 6.9 and 6.8, respectively. The DP of the ANTS-labeled conserved trimannosyl core substituted with fucose on lane 7 was 4.7. The separation of neutral ANTS-labeled neutral oligosaccharides on FACE was based on the hydrodynamic sizes of the oligosaccharides.

Based on the results of densitometric analysis, the relative amounts of asialo-oligosaccharides derived from the 3 different rHuEPO samples treated with NANase plus GALase were estimated to be as shown in Table 3. The oligosaccha-

Fig. 4. Mobility of ANTS-Labeled Oligosaccharide Standards and rHuEPO

Lane 1, oligoladder standards; lanes 2, 3, and 5, *N*-linked oligosaccharides from sample A, treated without an enzyme, and with NANase, and NANase+GALase, respectively. Lanes 4, 6 and 7, ANTS-labeled oligosaccharide standards.

Table 3. Relative Amounts of *N*-linked Oligosaccharide in Each EPO Sample Treated by NANase and GALase

Sample	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7
Sample A	4.4±0.7	16.9±1.0	3.0±1.3	43.9±0.4	14.8±0.3	16.6±0.8	1.3±0.1
Sample B	6.0±0.3	20.7±0.1	1.1±0.7	43.6±0.9	13.9±0.3	12.0±0.5	3.0±1.0
Sample C	3.8±2.0	15.2±2.1	8.9±2.0	40.6±4.8	13.6±4.3	6.6±0.7	11.2±1.9

Values are mean±S.D. of 5 experiments.

ride profiles for samples A and B were apparently different from that for sample C, as indicated by band 3, 6 and 7. Since the results were reproducible, pattern analysis of a NANase plus GALase-treated sample was useful for fingerprint analysis to assure the consistency of glycoprotein products.

DISCUSSION

Oligosaccharides are important contributors to the rate of clearance in circulation, biological activity, solubility, stability, antigenicity, and lectin binding properties of glycoproteins.²⁻⁴⁾ Terminal sialylation of oligosaccharides is also an important factor modulating the functions of various glycoproteins.²⁻⁴⁾

Rapid progress is being made in oligosaccharide structure determination and separation.^{3,4)} It is conceded that techniques for the routine analysis of oligosaccharide structures have been widely available. However, current technology, such as HPLC and HPAEC-PAD, does not yet lend itself to the routine determination of oligosaccharide structures.

In a previous paper we reported the optimum sialic acid content for the *in vivo* bioactivity of rHuEPO.¹⁰⁾ Takeuchi *et al.*¹⁷⁾ reported that rHuEPO with tetraantennary sugar chains possesses more potent activity *in vivo* than that with biantennary sugar chains. Therefore, higher branching of the *N*-linked sugar chains with the optimum sialic acid content is desirable for the effective expression of the *in vivo* biological

activity of EPO.

In the present study, we have examined the applicability of the FACE method to the analysis of three rHuEPOs, *i.e.*, two different EPOs from two types of CHO cells, and one EPO from BHK cells, as model proteins. We investigated suitable conditions for FACE sequencing of the oligosaccharides, so that comparison of the profiles of the three rHuEPOs with respect to sialo, asialo and agalacto oligosaccharides could be performed on the same gel.

Although FACE sequencing of *N*-linked oligosaccharides from glycoproteins has been reported,^{16,18)} in those studies the digestion was performed on an isolated oligosaccharide but not on an oligosaccharide mixture. Therefore, we first investigated the conditions under which all bands can be fully digested at the same time. Since purchased *N*-linked Oligosaccharide Sequencing Kits could not digest the bands fully because of the low activities of the enzymes, we performed a preliminary study with various kinds of NANase, GALase and HEXase, and found appropriate conditions.

We compared the profiling patterns and sequencing patterns of oligosaccharides from these three EPOs under appropriate conditions. Oligosaccharides that contain sialic acid migrate faster than neutral oligosaccharides. This is because the carboxyl groups in the sialic acid residues increase the net charge of the oligosaccharides, causing them to migrate faster in the gels. The separation of ANTS-labeled standard oligosaccharides on a FACE gel is based on the hydrodynamic sizes of the oligosaccharides. The order of mo-

Fig. 5. N-linked Oligosaccharide Structure Proposed from the DP Shift

bility of the standards was the conserved trimannosyl core substituted with fucose, and asialo-agalacto-biantennary- and asialo-agalacto-triantennary-, asialo-agalacto-tetraantennary, asialo-galactosylated biantennary-, asialo-galactosylated-triantennary-, and asialo-galactosylated tetraantennary-oligosaccharides.

Since the migration patterns of various oligosaccharides were highly reproducible, the method could be applied to compare the patterns at different steps of digestion of an oligosaccharide mixture, including sialo-, asialo- and agalacto-oligosaccharides, on the same gel.

Glycosylation of recombinant glycoproteins is affected by the cell culture conditions. Culture conditions are known to influence the phenotypic heterogeneity in glycosylation. Rothman *et al.*⁶⁾ reported that the glycosylation of immunoglobulin G was influenced by culture conditions, such as the pH of the culture medium and the proliferative state of the cells. Friedman and Higgins¹⁸⁾ reported that the level of sialylation was found to vary with the serum concentration and the culture method. Watson *et al.*¹⁹⁾ reported that rHuKallikrein produced by CHO cells under certain culture conditions contained reduced amounts of sialylated N-linked oligosaccharides. Therefore, it is more important at present that the glycosylation of a recombinant glycoprotein was shown to be consistent with the efficacy of a drug. Since, in the present study, the FACE method was indicated to be useful for evaluation of the consistency of the microheterogene-

ity of EPOs, we suggest that this method is also available for the evaluation of the glycosylation of other recombinant glycoproteins.

Oligosaccharide structures can be deduced by comparing the Dps (Degree of Polymerizations) of samples digested sequentially with exoglycosidase. As shown in Fig. 5, the upward shift of 4.6 units from DP=7.7 to DP=12.3 of tetraantennary oligosaccharides indicated that 4 sialic acids were released. Digestion of the starting material with NANase plus GALase caused a downward shift. The shift of -4.1 units indicates a loss of four Gal residues. Further digestion with these two enzymes plus HEXase resulted in only one band, which co-migrated with the authentic trimannosyl core substituted with fucose (Fig. 4, lane 7). The presence of the tetraantennary oligosaccharide with three N-acetylglucosamine units, which is the largest oligosaccharide molecule in EPO, could also be assumed, as shown in Fig. 5.

Starr *et al.*¹⁶⁾ reported that the rules for predicting a monosaccharide composition based on mobility shifts are as follows: NeuAc, Gal and GlcNAc were -1, 1 and 0.75, respectively. The present results showed good correspondence with the above reported values. This procedure was useful for quickly obtaining a great deal of structural information on the oligosaccharides in a mixture.

The availability of a rapid and relatively simple method for determining the carbohydrate structure in the development of glycoprotein drugs is a major challenge for glycobiologists,

manufacturers and regulatory agencies.^{4,19)}

The CCD response with the quantity of ANTS-labeled saccharides, including maltopentaose, glucose, and lactose were compared by Jackson.²⁰⁾ The results showed that the response of the ANTS-derivatives from *N*-glycosides are almost same. Quantification of carbohydrate bands was made by comparing the fluorescence intensity of a band with the ANTS-derivatives of glucose polymers with 3 glucose units to 9 glucose units by Hu and Vallee.²¹⁾ The results also indicated that FACE can be used to quantify the oligosaccharides from low pmol to 300 pmol. In the present study, the method was shown to have the detection limits as low as the low pmol range and to exhibit high reproducibility as to the high sensitivity of fluorescence. The results suggest that the FACE method will be useful for monitoring the states of glycosylation of secreted recombinant glycoproteins that are sensitive to changes in sialylation. Also, it can be used for the routine oligosaccharide profiling of glycoprotein products. Recently, Kumar *et al.*²²⁾ reported the elucidation of the *N*-linked oligosaccharide structure of recombinant human factor VIII by means of FACE.

Clearly, FACE is a rapid, sensitive, and potentially quantitative and technically readily available method for the routine analysis of oligosaccharides of glycoproteins.

Acknowledgements This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare Science Research Fund Subsidy granted by the Japan Health Science Foundation.

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