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Antibody-based enzyme-linked lectin assay (ABELLA) for the sialylated recombinant human erythropoietin present in culture supernatant

Hyoung Jin Kim, Seung Jae Lee, Hong-Jin Kim*

College of Pharmacy, Chung-Ang University, 221 Huksuk-Dong, Dongjak-Ku, Seoul 156-756, South Korea

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ABSTRACT

The terminal sialic acid of human erythropoietin (hEPO) is essential for *in vivo* activity. The current resorcinol and HPLC methods for analyzing α2,3-linked sialic acid require more than a microgram of purified rhEPO, and purification takes a great deal of time and labor. In this study, we assessed the use of an antibody-based enzyme-linked lectin assay (ABELLA) for analyzing non-purified recombinant hEPO (rhEPO). The major problem of this method was the high background due to terminal sialylation of components of the assay (antibody and bovine serum albumin) other than rhEPO. To solve this problem, we used a monoclonal antibody (Mab 287) to capture the rhEPO, and oxidized the bovine serum albumin used for blocking with meta-periodate. The sialic acid content of non-purified rhEPO measured by ABELLA was similar to that obtained by the resorcinol method on purified rhEPO. ABELLA has advantages such as adaptability and need for minimal amounts of rhEPO (40 ng/ml). Our observations suggest that ABELLA should reduce the time and labor needed to improve culture conditions so as to increase protein sialylation, and also facilitate the study of sialylation mechanisms.

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1. Introduction

Erythropoietin (EPO) is a glycoprotein that plays a major role in the proliferation and differentiation of erythrocytes from erythroid progenitor cells [1,2]. Human EPO (hEPO) consists of 166 amino acids and has three N-linked glycans (Ans 24, Asn 38, Asn 83) and one O-linked glycan (Ser 126). It has a molecular weight of 35–39 kDa [3], and its average carbohydrate content is approximately 40% [4].

Terminal sialylation is essential for the *in vivo* activity and stability of hEPO [5], which has a maximum of 14 terminal sialic acids (14 mole per mole EPO) [6]. Recombinant hEPO (rhEPO) has been produced in mammalian cell lines such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) because of the importance of its terminal sialylation [7]. CHO and BHK cells have only α2,3-linked sialic acids, unlike human cells that have both α2,3- and α2,6-linked sialic acids [8]. The terminal sialylation of glycoproteins produced in CHO or BHK cells is affected by numerous factors that include ammonium accumulation, addition of sodium butyrate or amino acids, culture period and culture temperature [2,3,9–13].

Previous methods for analyzing sialylation, such as enzyme-linked lectin assay (ELLA), resorcinol and HPLC method require purified glycoprotein [12–16]. In addition, the resorcinol method requires more than a microgram of purified rhEPO [17,18]. The purification procedure takes a great deal labor and time. The lack of a convenient method for analyzing the sialylation limits the investigation of sialylation changes in response to various culture conditions and of sialylation mechanism.

In this study we assessed the use of an antibody-based enzyme-linked lectin assay (ABELLA) for analyzing non-purified rhEPO directly in culture supernatants. In this method, 96-well microtitre plates were coated with anti-hEPO antibody to capture the rhEPO, and terminal sialic acid was detected using a lectin. To optimize condition of ABELLA, we carefully selected the anti-hEPO antibody and improved the blocking conditions. The result for terminal sialic acid content obtained by ABELLA was compared with that from the traditional resorcinol method in order to test the utility of the new method.

2. Materials and methods

2.1. Preparation of asialo EPO

Terminal sialic acids were removed with proteomic grade α(2 → 3,6,8,9) neuraminidase kit from *Arthrobacter ureafaciens*, with 5× reaction buffer (Sigma, St. Louis, MO, USA). Recormon was prepared at 1.7 mg/ml in 1× reaction buffer. One hundred μl of Recormon (170 μg per reaction; Roche, Indianapolis, Indiana, USA)

* Corresponding author. Tel.: +82 2 820 5613; fax: +82 2 816 7338.
E-mail address: hongjink@cau.ac.kr (H.J. Kim).

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and 4 μl of neuraminidase (2 Sigma units) were mixed and incubated at 37 °C for 3 h. The reactions were stopped by heating for 5 min at 100 °C. The solutions were dialyzed against distilled water to remove remaining sialic acids and removal of terminal sialic acids was confirmed by analysis of terminal sialylation using lectin, as described in [14,15]. The concentration of asialo EPO was determined with a BCA protein reagent kit.

2.2. Sialylation analysis of anti-hEPO antibodies

α2,3- and α2,6-terminal sialylation of the anti-hEPO monoclonal antibody (mAb) and of the polyclonal antibody (pAb) was determined using biotinylated *Maackia amurensis* lectin II (MAA II, Vector Laboratories, Burlingame, CA, USA) and biotinylated *Sambucus nigra* lectin (SNA, Vector Laboratories), respectively [19]. Mab 287 (R&D systems, Minneapolis, MN, USA) and anti-hEPO rabbit IgG (R&D systems) were used as anti-hEPO mAb and pAb, respectively. A 96-well microtitre plate was coated overnight at 4 °C with 1.6 μg of sample per well. Unbound proteins were removed by washing three times with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), and the plates were blocked with TBS containing 1% Tween 20 for 1 h at room temperature (RT). After washing twice with TBST, the biotin-labeled lectins were added and reacted at RT for 1 h. Then the plates were washed four times with TBST, and streptavidin–HRP (Pierce, Rockford, IL, USA) was added and incubation continued at 37 °C for 30 min [19]. Color reactions were developed using *o*-phenylenediamine (Sigma) and measured at 492 nm.

2.3. Cell culture

The rhEPO-producing CHO (rhEPO-CHO) cells were maintained as an adherent cultures in Minimum Essential Medium Alpha (MEM-α; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (Gibco BRL;10,000 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in 0.85% saline), and 25 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulfonic acid). rhEPO-CHO cells were cultured at 37 °C and in 5% CO_2. Ammonium chloride (NH_4Cl) was used as a sialylation inhibitor [3,20]. rhEPO-CHO cells were seeded at 5.4×10^6 cells in T-175 flask and cultured for 3 d in medium containing 0, 7, 15, and 30 mM NH_4Cl.

2.4. Determination of rhEPO concentration

The amount of rhEPO present in culture supernatants was determined by sandwich ELISA using Recormon (Roche) as a standard. For the sandwich ELISA, anti-hEPO mAb (R&D Systems) was used as a capture antibody, and anti-hEPO pAb (R&D Systems) together with HRP-conjugated goat anti-rabbit IgG (Sigma) was used for detection of rhEPO. A 96-well microtitre plate was coated overnight at 4 °C with 100 ng of anti-hEPO mAb. Unbound proteins were removed by washing three times with TBST and the plate was blocked with 2% bovine serum albumin (BSA) in TBST for 1 h at RT. 0, 0.5, 1, 2, 4 and 8 ng/ml of Recormon (5000 IU, Roche) were prepared for standard reference curve. Sample and standard solutions were added into their respective wells and incubated for 2 h at 37 °C. After washing three times with TBST, anti-hEPO pAb was added and incubated at 37 °C for 30 min. The plate was washed four times with TBST, and anti-rabbit IgG was added and incubated at 37 °C for 30 min. Color reactions were developed using *o*-phenylenediamine (Sigma) and measured at 492 nm [21]. The concentration of purified rhEPO was determined with a BCA protein reagent kit (Pierce).

2.5. Preparation of oxidized BSA

BSA fraction V (Sigma) was oxidized by dissolving it in 20 mM meta-periodate (Sigma), 50 mM sodium acetate, pH 4.0 buffer and leaving at 4 °C for 30 min. After the oxidation with periodate, the mixture was dialyzed against TBS (pH 7.4), and Tween 20 was added to the final concentration of 0.1%.

2.6. Antibody-based enzyme-linked lectin assay (ABELLA)

Anti-hEPO mAb was prepared at 4 μg/ml in 15 mM sodium carbonate anhydrous, 35 mM sodium hydrogen carbonate and 3 mM sodium azide, pH 9.6 buffer. A 96-well microtitre plate (Greiner, Frickenhausen, Germany) was coated overnight at 4 °C with 400 ng (100 μl) of anti-hEPO mAb (Mab 287, R&D Systems) per well. Unbound proteins were removed by washing three times with TBST (pH 7.4) and the plate was blocked with 300 μl of 2.5% oxidized BSA (see preparation of oxidized BSA) in TBST for 3 h at RT. After washing three times with TBST, the plate was incubated with rhEPO at RT for 1 h. To capture the rhEPO present in culture supernatant, the supernatants were diluted with 0.3% oxidized BSA in TBST (40 ng/ml of rhEPO) and 100 μl aliquots were added to the wells. After washing three times with TBST, 100 μl of MAA II (prepared at 1.5 μg/ml, Vector Laboratories) was added per well and the plates were left at RT for 1 h. They were then washed four times with TBST, and 100 μl of streptavidin–HRP (prepared at 200 ng/ml, Pierce) was added per well and incubated at 37 °C for 30 min. MAA II and streptavidin–HRP were diluted with TBST for detection of the sialic acid. After washing five times with TBST, color reactions were developed using *o*-phenylenediamine (Sigma) and measured at 492 nm.

2.7. Analytical validation of ABELLA

To determine limit of detection (LoD) and limit of quantification (LoQ), MEM-α plus 10% FBS was diluted 1:300 with 0.3% oxidized BSA, and the diluents were measured as the zero calibrator. To determine LoD and LoQ, each measurement was assayed as duplicate. LoD was calculated as the mean absorbance value of quintuplicate measurement of zero standards plus three times the standard deviation (S.D.). LoQ was calculated as the mean absorbance value of quintuplicate measurement of zero standards plus 10 times the S.D. LoD and LoQ were calculated from the standard curve. The inter-assay precision and accuracy (between-day repeatability precision and accuracy) was determined in five replicates for three levels of mole sialic acid per mole EPO. rhEPO was prepared at 12, 6 and 3 μg/ml in MEM-α plus 10% FBS and diluted 1:300 with 0.3% oxidized BSA in TBST. Dilution linearity was determined in four replicates of predetermined rhEPO. The rhEPO was prepared at 12 μg/ml in MEM-α plus 10% FBS and diluted with 0.3% oxidized BSA in TBST (40 ng/ml of rhEPO). This spiked rhEPO was serially diluted with 0.3% oxidized BSA in TBST and then assayed.

2.8. Purification of rhEPO

The culture supernatant of rhEPO-CHO was dialyzed against equilibration buffer (10 mM $NaH_2PO_4 \cdot 2H_2O$, 50 mM NaCl, pH 6.0) at 4 °C. 20 ml of dialyzed sample was applied to a HiTrap™ Heparin HP column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the column was washed with equilibration buffer at a flow rate of 1 ml/min. Heparin binding proteins were eluted with a linear gradient of NaCl (from 0.05 to 1 M), and rhEPO-containing fraction were identified by sandwich enzyme-linked immunosorbent assay (ELISA) as described above.

An immuno-affinity column was prepared by coupling 1.5 mg of anti-hEPO (R&D System) with CNBr-activated Sepharose-4 fast flow

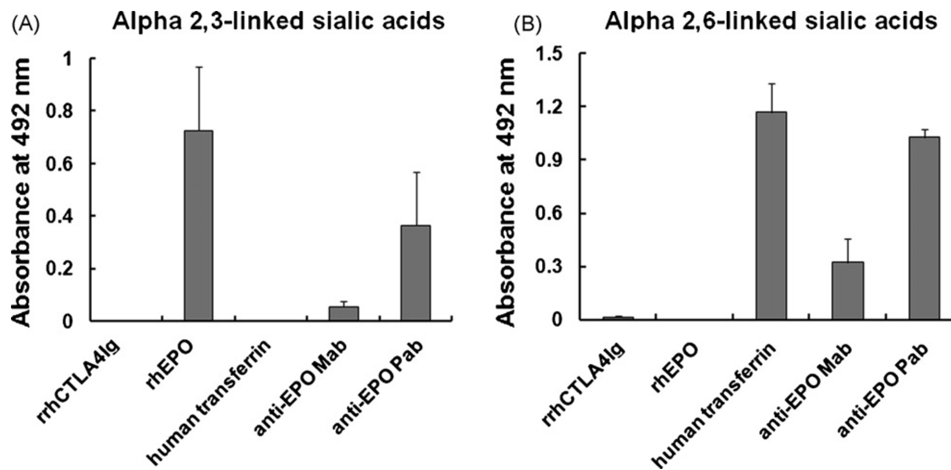

Fig. 1. Terminal sialylation of anti-hEPO mAb and pAb. α2,3-linked sialic acids were detected using biotinylated *Maackia amurensis* Lectin II (MAA II) (A), and α2,6-linked sialic acids were detected using biotinylated *Sambucus nigra* (SNA) (B). The α2,3-linked sialic acid contents of the anti-hEPO mAb and pAb were compared with negative controls (rrhCTLA4Ig and human transferrin) and a positive control (rhEPO), whereas their α2,6-linked sialic acid contents were compared with negative controls (rrhCTLA4Ig and rhEPO) and a positive control (human transferrin). Values are means ± S.D. of triplicate measurements.

gel (Amersham Pharmacia Biotech), and washing with phosphate-buffered saline (PBS), pH 7.4, containing 50 mM NaCl. The rhEPO fractions from the heparin chromatography were recirculated 20 times with the aid of a peristaltic pump. The gel was washed with PBS, pH 7.4, containing 50 mM NaCl, and the rhEPO was eluted with 0.1 M glycine, pH 2.8. The eluted fractions were rapidly neutralized with 1 M Tris–Cl, pH 9.0.

2.9. SDS-PAGE

All samples were subjected to 12% PAGE in the presence of sodium dodecyl sulfate according to the method of Laemmli under reducing conditions [22]. Separated proteins were visualized by silver staining (Amersham Pharmacia Biotech).

2.10. Quantitative analysis of sialic acid (resorcinol method)

To remove free sialic acids, purified rhEPO was dialyzed against distilled water. The sialic acid released by hydrolysis of the purified rhEPO was measured by the resorcinol method as described in [17,18].

3. Results

3.1. Terminal sialylation of anti-hEPO mAb and anti-hEPO pAb

Terminal sialylation of the antibody used in ABELLA adds to the background of the assay [23]. Therefore, the α2,3- and α2,6-linked sialic acid content of the anti-hEPO mAb and pAb were investigated. Rice cell-derived recombinant CTLA4Ig (rrhCTLA4Ig), which is known to have no terminal sialic acids [14], was used as a negative control. rhEPO has only α2,3-linked sialic acid [8], and human transferrin contains only α2,6-linked sialic acid [24]. Therefore, these were used as positive controls for α2,3- and α2,6-sialylation, respectively. Virtually no α2,3-linked sialic acid was detected in the anti-hEPO mAb (Fig. 1A), whereas the anti-hEPO mAb has a α2,6-linked sialic acid content (Fig. 1B). High levels of both α2,3- and α2,6-linked sialic acids were found in the anti-hEPO pAb (Fig. 1A and 1B). These results indicated that the anti-hEPO mAb dose not possess α2,3-linked sialic acids that could affect the background in the ABELLA. Therefore, anti-hEPO mAb was used as capture antibody.

3.2. Effect of blocking agents on the performance of ABELLA

BSA is used as a blocking agent in ELISAs to minimize non-specific reactions. To investigate the background due to blocking agent in ABELLA, a 96-well microtitre plate was coated with anti-hEPO mAb and blocked with 1% Tween 20, and either 1% BSA or 1% oxidized BSA for 3 h at RT. MAA II and streptavidin–HRP were added successively, and the color reactions were developed as described in Section 2. As shown in Fig. 2, the OD level given by 1% Tween 20 was less than 0.2, whereas that given by 1% BSA was 0.8. The purity of the BSA was about 96%. This result indicates that the BSA fraction contains sialoglycoproteins. To remove the effect of the sialoglycoproteins, the BSA was oxidized with meta-periodate for 30 min as described in Section 2. As a result, the OD level of the oxidized BSA decreased to that of 1% Tween 20 (Fig. 2). This indicates that oxidation of the BSA is essential for sialylation analysis using ABELLA.

3.3. Specificity of the ABELLA

To confirm the specificity of ABELLA for rhEPO sialylation, the ODs of a blank (TBST), medium, medium + asialo EPO, medium + human transferrin and medium + rhEPO were measured

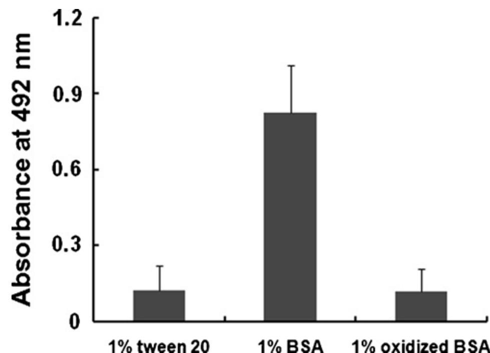

Fig. 2. The effect of blocking agents on the performance of ABELLA. To investigate the background of blocking agent, a 96-well microtitre plate was coated with anti-hEPO mAb (400 ng per well), and the plate was blocked with 1% Tween 20 in TBST, 1% BSA in TBST or 1% oxidized BSA in TBST for 3 h at RT. The oxidized BSA was prepared as described in Section 2. Backgrounds were detected with MAA II and streptavidin–HRP. Values are means ± S.D. of triplicate measurements.

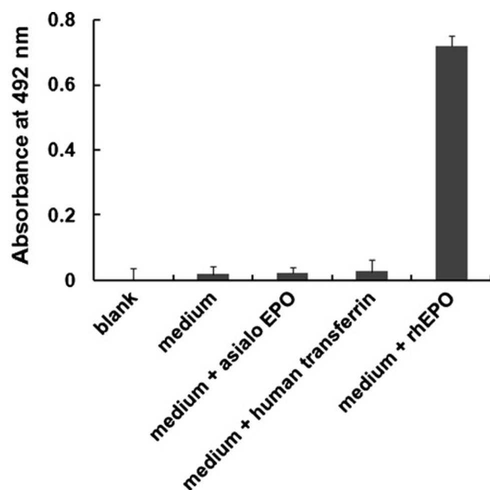

Fig. 3. Specificity of the ABELLA for sialylated rhEPO. A 96-well microtitre plate was coated and blocked as described in Section 2. The blank was incubated with TBST. Medium (MEM-α plus 10% FBS) was diluted 1:300 with 0.3% oxidized BSA in TBST. Asialo EPO, human transferrin and rhEPO were prepared at concentrations of 12 μg/ml in medium containing 10% FBS, and samples were then diluted 1:300 with 0.3% oxidized BSA in TBST (40 ng/ml). After incubation for 1 h, ODs were developed with MAA II and streptavidin–HRP. Values are means ± S.D. of triplicate measurements.

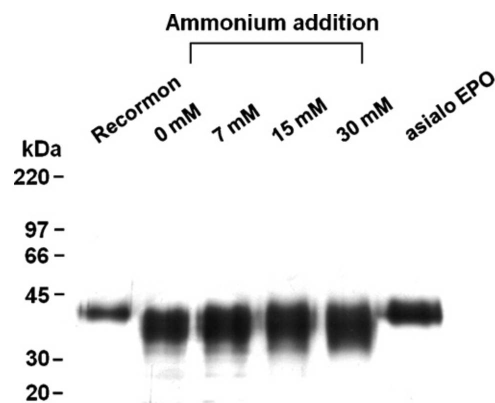

Fig. 4. SDS-PAGE analysis of purified rhEPO. rhEPO-CHO cells were seeded at 5.4×10^6 cells into T-175 flasks and cultured for 3 d in medium containing 0, 7, 15 and 30 mM NH_4Cl. rhEPO in the culture supernatant was purified as described in Section 2. The purity and molecular weight of purified rhEPO was compared with Recormon and asialo EPO on an SDS-PAGE gel.

(Fig. 3). The MEM-α plus 10% FBS was diluted 1:300 with 0.3% oxidized BSA in TBST. Asialo EPO, human transferrin and rhEPO were prepared at 12 μg/ml in MEM-α containing 10% FBS and diluted 1:300 (40 ng/ml) in 0.3% oxidized BSA in TBST. The ODs of medium, medium + asialo EPO, medium + human transferrin were similar to the OD of the blank, whereas the OD of the medium + rhEPO was 0.7 (Fig. 3). Since the MEM-α with 10% FBS contains various glycoproteins, this result indicates that the ABELLA does not recognize the glycoproteins in FBS, human transferrin or asialo EPO, and hence is specific for the terminal sialic acids found in rhEPO (Fig. 3).

3.4. Evaluation of the ABELLA

LoD, LoQ, precision and accuracy of the ABELLA were determined as described in Section 2. LoD and LoQ were 0.27 and 0.81 mole sialic acid/mole EPO, respectively. Inter-assay coefficients of variation (CV) were 11.2–8%, and inter-assay recoveries were 89.7–101.8% (Table 1). Observed mole sialic acid per mole EPO according to serial dilution was similar with expected mole sialic acid per mole EPO (Table 2). CV according to serial dilution were 16.1–3.3%, and the recoveries were 86.3–101.5% (Table 2). These results indicate that the ABELLA is suitable for analyzing the terminal sialylation of rhEPO present in culture supernatant.

3.5. Comparative analysis of sialylation by the resorcinol method and ABELLA

3.5.1. Analysis of sialylation by the resorcinol method

To inhibit terminal sialylation, NH_4Cl was used as a sialylation inhibitor [3]. rhEPO-CHO cells were cultured for 3 d in medium containing 0, 7, 15 and 30 mM NH_4Cl. The rhEPO produced in each culture was purified, and its purity was confirmed by SDS-PAGE (Fig. 4). The amounts of the purified rhEPO, asialo EPO and Recormon were determined with a BCA protein reagent kit as described in Section 2. The sialic acid contents of the purified rhEPO, asialo EPO and Recormon were then determined by the resorcinol method (Fig. 5A). The sialic acid contents of the Recormon and asialo EPO were 13.3 and 0.7 mole per mole EPO, respectively, and the sialic acid content of the rhEPO purified from the culture supernatant decreased as a function of increasing NH_4Cl concentration (Fig. 5A).

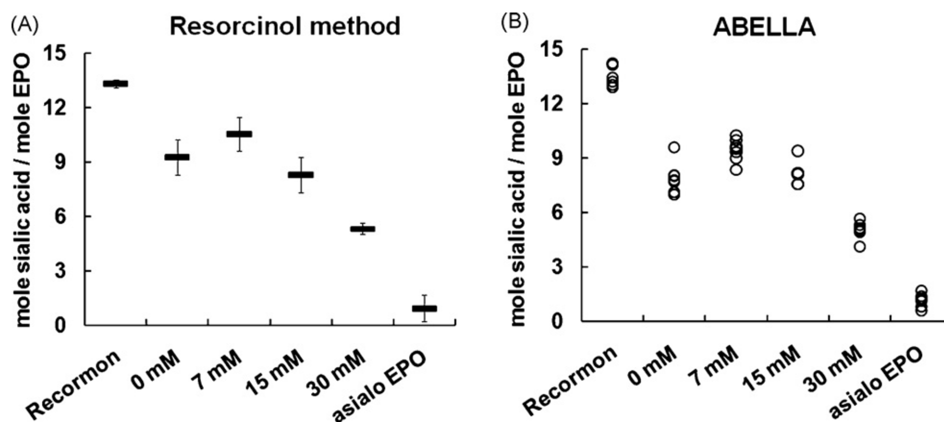

Fig. 5. Sialic acid quantification using the resorcinol method and ABELLA. The sialic acid contents of purified rhEPO and of rhEPO in the culture supernatant were determined by the resorcinol method (A) and ABELLA (B), respectively. Recormon and asialo EPO were prepared at concentrations of 12 μg/ml in medium, and the samples were then diluted with 0.3% oxidized BSA in TBST to a concentration of 40 ng/ml for the ABELLA method. The other samples (NH_4Cl added) were also diluted with 0.3% oxidized BSA in TBST to a concentration of 40 ng/ml. Recormon, purified rhEPOs and asialo EPO were prepared at 150 μg/ml and measured by the resorcinol method as described in Section 2. Values of resorcinol method are means ± S.D. of duplicate measurements. Values of ABELLA are expressed as points of seven independent assays.

Table 1
Inter-assay precision and accuracy of ABELLA

Expected (mole sialic acid/mole EPO)	*N*	Observed (mole sialic acid/mole EPO)	S.D.	CV (%)	Recovery (%)
Low (3.3)	5	2.9	0.3	11.2	89.7
Medium (6.65)	5	6.7	0.5	7.4	101.8
High (13.3)	5	13	1	8	98.2

Table 2
Dilution linearity of ABELLA

Dilution factor	Expected (mole sialic acid/mole EPO)	*N*	Observed (mole sialic acid/mole EPO)	CV (%)	Recovery (%)
1	13.3	4	12.7	3.3	95.1
2	6.6	4	6.0	11.4	90.5
4	3.3	4	2.9	8.4	86.3
8	1.6	4	1.6	16.1	101.5

3.5.2. Sialylation analysis using ABELLA

To measure sialic acid content using the ABELLA, the amounts of rhEPO produced in cultures in the presence of 0, 7, 15 and 30 mM NH_4Cl were determined by mean of quadruplicate ELISAs, and CV of the ELISA was from 8.1% to 4.4%. rhEPO was assayed at 40 ng/ml for the ABELLA. As shown in Fig. 4, the Recormon and asialo EPO were purified samples. Therefore, to make condition such as non-purified rhEPO, the Recormon and asialo EPO were prepared at concentrations of 12 μg/ml in MEM-α containing 10% FBS and diluted at 40 ng/ml with 0.3% oxidized BSA in TBST. The average sialic acid content of Recormon is known to be 13 mole per mole EPO [6]. The Recormon was prepared at 50, 25, 12.5, 6.25, 3.1 and 0 ng/ml to generate a standard curve, and these concentrations present 16.2, 8.1, 4, 2, 1 and 0 mole per mole EPO, compared to 40 ng/ml of Recormon, asialo EPO and rhEPO produced in cultures in the presence of NH_4Cl, respectively. The standard curve of OD versus sialic acid content was almost linear ($R^2 = 0.996$, data not shown), and the sialic acid contents measured by ABELLA of Recormon, asialo rhEPO and rhEPO produced in cultures in the presence of NH_4Cl were similar to those determined by the resorcinol method (Fig. 5B). This result indicates that the sialic acid content of rhEPO can be determined by ABELLA even if the rhEPO is not purified.

4. Discussion

In this study, optimum conditions for rhEPO-specific ABELLA were suggested, and the possibility of using it for sialylation analysis was confirmed by comparing it with the traditional resorcinol method. In a previous report, it was shown that the sialic acids present in antibodies create a major problem for sialylation assays of human transferrin. Gornik and Lauc [23] analyzed the sialylation of human transferrin in serum using a combination of anti-human transferrin antibody and lectin, and oxidized the antibody to reduce the background due to its sialic acids. In this study, it was confirmed that anti-hEPO mAb has almost no α2,3-linked sialic acids (Fig. 1) and that its affinity for hEPO decreased when it was oxidized (data not shown).

We found that, contrary to a previous report [23], BSA in the blocking buffer generated a high background OD that was a major problem (Fig. 2), and the same was true of skim milk-based blocking buffer (data not shown). Use of BSA or skim milk is essential to minimize non-specific antibody reactions [25]. To reduce the high background, BSA was oxidized with meta-periodate and dialyzed against TBS. This method proved to be very effective in reducing the background (Fig. 2). The purity of commercial BSA fractions is 96–98%. In this study, it was confirmed that the high background of BSA was caused by α2,3-linked sialoglycoproteins in the BSA fraction. In addition, we confirmed that the BSA fraction has α2,6-linked sialoglycoproteins and that the oxidization was also very effective in reducing the non-specific reaction of the α2,6-linked sialic acids (data not shown). The glycoform of the glycoprotein is known to be heterogeneous [8], and sialic acids are linked into termini of the glycans. Therefore, existence of the sialoglycoproteins in the BSA fraction indicates the existence of other glycoforms such as galactosylation, mannosylation and fucosylation. When using an antibody–lectin combination such as ABELLA, it seems that the existence of glycoproteins in the blocking agent should be taken into account in the analysis of other carbohydrates as well as terminal sialylation.

In this study, the concentrations of rhEPOs present in culture supernatants were determined by mean of quadruplicate ELISAs, and ELISA CV was less than 10%. For the accuracy and precision of sialylation analysis using the ABELLA, reproducibility of the ELISA is important.

Conventional methods of sialylation analysis require purification of the recombinant glycoproteins, and the resorcinol and HPLC methods both require more than a microgram of purified protein [17,18]. This purification requirement limits investigations of sialylation patterns and sialylation mechanisms. The average sialic acid content of the rhEPO produced in CHO cells is about 10 mole per mole EPO [12,18]. However, the European Pharmacopoeia requires determination of the amount of sialic acids, which has to exceed 10 mole per mole EPO [6]. Therefore, a considerable fraction of overall rhEPO production cannot be marketed for drug use [6]. In this study, we successfully assayed the sialylation of rhEPO at 40 ng/ml by ABELLA (Tables 1 and 2; Fig. 5). Therefore, sialylation analysis is possible using minimal amounts of rhEPO, purification is not necessary, and sialylation can easily be studied as a function of culture conditions. We anticipate that rhEPO-specific ABELLA will reduce the cost, time and labor involved in varying culture conditions to increase the terminal sialylation of rhEPO and will permit efficient quality control of rhEPO.

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