J. Microbiol. Biotechnol. (2001), **11**(6), 1087–1092

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Effect of Sodium Butyrate on Glycosylation of Recombinant Erythropoietin

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Received: November 5, 2001
Accepted: December 7, 2001

Abstract The effect of Sodium Butyrate (NaBu) on the N-linked oligosaccharide structure of Erythropoietin (EPO) was investigated. Recombinant human EPO was produced by CHO cells grown in an MEMα medium with or without 5 mM NaBu, and purified from the culture supernatants using a heparin-sepharose affinity column and immunoaffinity column. The N-linked oligosaccharides were released enzymatically and isolated by paper chromatography. The isolated oligosaccharides were then labeled with a fluorescent dye, 2-aminobenzamide, and analyzed with MonoQ anion exchange chromatography and GlycosepN amide chromatography for the assignment of a GU (glucose unit) value. A glycan analysis by HPLC showed that the most significant characteristic effect of NaBu was a reduction in the proportion of glycans with tri- and tetrasialylated oligodaccharides from 21.30% (tri-) and 14.86% (tetra-) in the control cultures (without NaBu) to 8.72% (tri-) and 1.25% (tetra-) in the NaBu-treated cultures, respectively. It was also found that the proportion of asialo-glycan increased from 12.54% to 23.69% when treated with NaBu.

Key words: Erythropoietin, sodium butyrate, glycosylation

EPO is a glycoprotein produced by the kidney to control the production of erythrocytes in mammals. Human EPO has four glycosylation sites: a single O-linked and three N-linked sites, and carbohydrates comprise of 40% molecular weight [1, 2]. It has been previously shown that the N-linked sugar chains of EPO play important roles in solubility, cellular processing and secretion, and *in vivo* bioactivity [3]. Incomplete glycosylation of N-linked oligosaccharides leads to decrease in activity *in vivo* [3, 4, 5]. Consequently, the well-branched structure of the N-linked chain is suggested to play a role in maintaining a higher plasma level, which provide an effective transfer to a target organ and stimulates in erythroid progenitor cells. The importance of terminal sialic acid residues in the expression of biological activity has also been reported using a crude EPO preparation [6]. By the enzymatic removal of terminal sialic acids from the oligosaccharides of uhEPO or rhEPO, galactose residues become exposed and bind to specific hepatocyte lectins, which in turn led to the prompt removal of the EPO molecules from the plasma [6, 7, 8]. Therefore, the removal of terminal sialic acids from EPO destroys its *in vivo* activity and desialylated EPO is cleared from the circulation faster than unmodified EPO.

Sodium butyrate (NaBu) is known to enhance gene expression in mammalian cells and has been used in the mass production of recombinant proteins in recombinant CHO cells [9, 10, 11, 12]. However, since the oligosaccharide structure of EPO is crucial for its *in vivo* biological activity, the influence of butyrate on the glycosylated recombinant EPO needs to be evaluated. The current authors recently established a highly sensitive, accurate, and quantitative method for analysing oligosaccharide structures [13]. The present work therefore, analyzed the effect of butyrate on the recombinant EPO N-linked oligosaccharide structure.

Materials and Methods

Cell Line and Culture Conditions

The recombinant erythropoietin (EPO)-producing Chinese hamster ovary (CHO) cell line (EC-1) was kindly provided by Dr. H. J. Hong from the KRIBB (Korea Research Institute of Bioscience and Biotechnology). The cell line was constructed by the introduction of cDNA encoding human EPO under the control of a Cytomegalovirus (CMV) promoter. The cells were cultured in MEMα

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supplemented with 10% dFBS, and 3.5 g/l glucose containing the antibiotic- antimycotic solution in T-185 flasks under an atmosphere of humidified 5% CO_2 at 37°C. After being cultured under the 10% dFBS conditions for 3 days, in the case of the control (without NaBu), the culture medium was replaced with MEMα containing 1% dFBS and the cells were cultured for one more day. Whereas in the case of the NaBu treated culture, the culture medium was replaced with MEMα containing 1% dFBS and 5 mM NaBu, then the cells were cultured for another 12 h.

Purification of Recombinant EPO

An EC-1 conditioned medium was applied to a 1×12 cm, Heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech, Uppsala, Sweden), which had been previously equilibrated with the buffer (10 mM sodium phosphate, pH 6.0, 50 mM NaCl). The proteins were eluted applying a salt gradient at a flow rate of 1 ml/min, using an FPLC system. The elution of EPO was analyzed using the ELISA method.

The EPO-containing fractions recovered from the Heparin-sepharose column were pooled and equilibrated with PBS. This preparation was applied to an immunoaffinity (CNBr-activated sepharose 4B coupled with monoclonal anti human EPO) column and eluted. The purified EPO was further evaluated by SDS-PAGE.

Preparation of N-linked Oligosaccharides from Recombinant EPO

The purified EPO was dialyzed in distilled water and freeze-dried in a microtube. About 100 μg of EPO was then dissolved in 50 μl of a reaction buffer (50 mM sodium phosphate, pH 7.0, 12.5 mM EDTA, 0.1% SDS) and boiled for 10 min for the denaturation of the protein. Thereafter, 2 μl of Triton X-100 and 2 units of N-glycosidase F were treated and incubated for 24 h at 37°C. The oligosaccharides were recovered by a Sep-Pak C_{18} cartridge (Waters, Milford, MA, U.S.A.). The samples were eluted in 5% of acetonitrile, pooled, and freeze-dried.

2-Aminobenzamide(2-AB) Labeling

The 2-AB labeling of the oligosaccharides was performed according to the method of Brigge *et al.* [14]. To an aspirated oligosaccharide sample, 5 ml of 0.25 M 2-aminobenzamide in acetic acid-DMSO (3:7 v/v) containing 1.0 M sodium cyanoborohydride was added and the mixture was incubated for 2 h at 65°C. After the incubation, the mixture was applied to a paper chromatography eluted with a buffer (n-butanol : ethanol : distilled water=4:1:1), extracted, and finally aspirated for an HPLC analysis.

HPLC Analysis of Oligosaccharides

A strong anion-exchange column was used to separate the neutral oligosaccharides from the acidic oligosaccharides. The oligoaccharide samples were dissolved in distilled water and applied to a MonoQ HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). After eluting the neutral oligosaccharides with 10 ml of water, the acidic oligosaccharides were eluted with a 1 M gradient of ammonium acetate, pH 4.0, at a flow rate of 1 ml/min at room temperature.

The peaks from the MonoQ were collected, aspirated, and freeze-dried to analyze in a Glycosep-N column (Oxford GlycoScience, Oxford, U.K.) according to the glucose unit (GU). The acetonitrile and 50mM ammonium acetate (pH 4.0) ratio was changed linearly from 80:20 to 47:53 (v/v) over 160 min after injection at a flow rate of 1.0 ml/min at 30°C.

RESULTS AND DISCUSSION

Purification of Recombinant EPO

The culture supernatant from each culture was withdrawn, filtered, and the pH equilibrated to 6.0. Supernatant was primarily eluted through a Heparin-sepharose column. The fractions containing EPO collected from the Heparin-sepharose were then applied to an immunoaffinity column. The SDS-PAGE profile in Fig. 1 shows that the recombinant EPO had a high purity. The amount of protein was adjusted to 30 ug in each lane to compare the purity of the eluants.

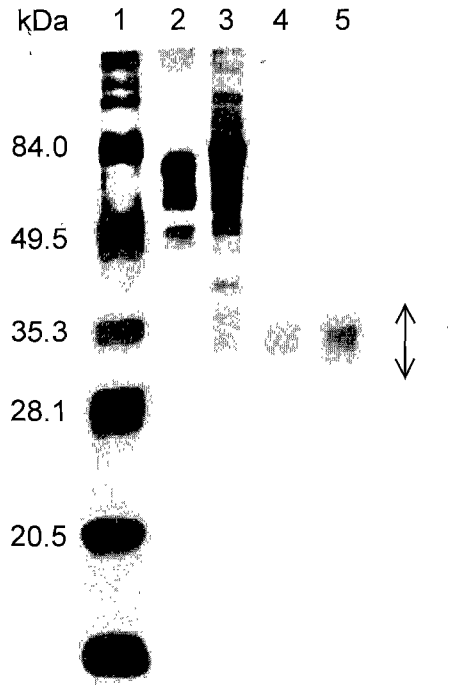

Fig. 1. SDS-PAGE of fractions from each step in purification of Recombinant EPO.

Lane 1, size marker; lane 2, cell supernatant; lane 3, heparin sepharose pool; lane 4, Immunoaffinity pool (NaBu-treated); lane 5, Immunoaffinity pool (control).

Since the volume and content of protein in the cultured medium were too large to load to the immunoaffinity chromatography, an and additional step was needed prior to the immunoaffinity chromatography to reduce the total protein contents by removing the contaminated proteins.

Heparin affinity chromatography greatly facilitates the purification of various growth factors and cytokines, including bFGF (basic fibroblast growth factor), PDGF (platelet-derived growth factor), EGF (epidermal growth factor), IL-2 (interleukin-2), IFN-γ (interferon-gamma), and many others [15, 16, 17, 18]. Although heparin is a polysaccharide with long, linear, and highly sulfated chains, based on an N-acetylglucosamine-uronic acid disaccharide repeat, these chains are extremely heterogeneous in structure. It has already been established that proteins bind with heparin through a heparinoid-protein interaction involving the basic amino acid sequence of the protein [19]. Since EPO is a growth factor of hematopoietic cells, similar to other heparin-binding growth factors containing a basic portion of protein (deglycosylated EPO's p*I*=9.2), heparin affinity column was used as the first step of purification.

The immunoaffinty chromatography had an attached ligand which was an antibody specific to EPO. As such, the column only retained the EPO that bound to the ligand attached to the beads, while the remaining proteins just passed through the column. Consequently, the EPO was purified from the other contaminating proteins.

Release of Oligosaccharides of EPO and 2-Aminobenzamide Labeling

We have previously reported that the hydrazinolysis method allows for the rapid and complete cleavage of glycans [13]. However, since a hydrazinolysis reaction react on the glycan linkage that results in a partial loss of sialic acid, it was not appropriate for a highly sialylated glycoprotein, such as EPO. Therefore, a simple enzymatic method and free from any hazardous reaction conditions was used to cleave the oligosaccharides of EPO.

There are two types of fluorescence labeling methods in the analysis of an oligosaccharide structure; the pyridylamine (PA) labeling method and the 2-aminobenzamide labeling method. Kanazawa *et al.* reported that sialic acid is partially lost when using the PA labeling method, as compared with the 2-AB labeling method [20]. Furthermore, when using the PA labeling method, the reagent peak obtained with anion-exchange (DEAE) column chromatography overlaps with the asialo oligosaccharide peak around the void volume. Consequently, the 2-AB labeling method was used for the fluorescence labeling.

Comparative Study of Oligosaccharide Structure from EPO Produced in Culture with or without NaBu

The labeled oligosaccharide samples were subjected to MonoQ column chromatography at pH 4.0. Since MonoQ is a strong anionic-exchange column, the oligosacharides were eluted according to the negative charge due to their sialic acid content. Asialo neutral oligosaccharides were eluted at the beginning of the elution, and then as the gradient formed with 1 M ammonium acetate, more sialylated oligosaccharides were eluted. The degree of sialylation of the oligosaccharides from the EPO in the control and the NaBu-supplemented culture was analyzed and compared (Fig. 2). The chromatogram showed a significant degree of variation in the oligosaccharide patterns. The relative amount of sialylated oligosaccharides was quantified by the peak areas (Table 1). The amounts of neutral(asialo) oligosaccharides was higher in the NaBu sample compared to the control. The most significant characteristic of the sample containing NaBu was that the relative amounts of tri- and tetrasialylated oligosaccharides were reduced from 21.30% (tri-) and 14.86%

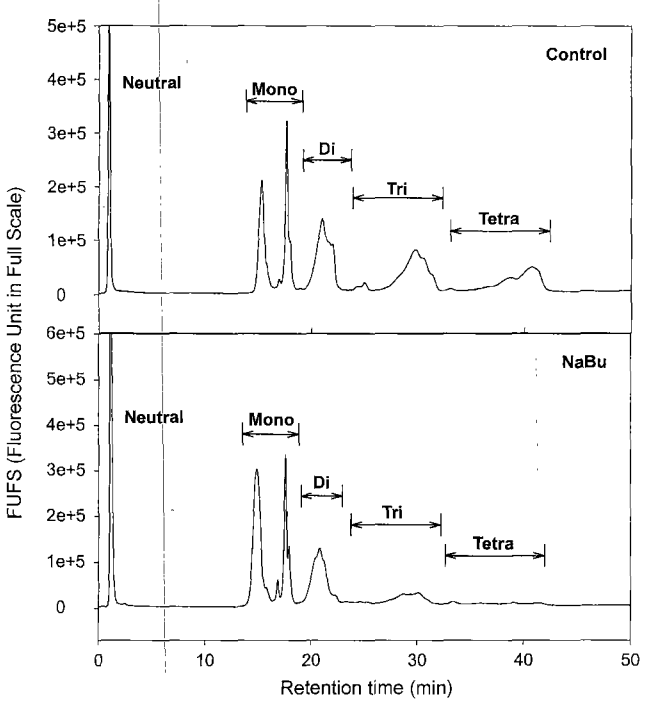

Fig. 2. MonoQ chromatogram of oligosaccharide from control and NaBu-treated sample.
The arrows and ranges indicate the distribution of sialylated oligosaccharides.

Table 1. Relative amount of sialylated oligosaccharides in control and NaBu-treated sample.

Sialylated oligosaccharides	Control (Relative amount in %)	Sample treated with NaBu (Relative amount in %)
Neutral (asialo)	12.54	23.69
Monosialylated	28.80	44.57
Disialylated	22.50	21.77
Trisialylated	21.30	8.72
Tetrasialylated	14.86	1.25

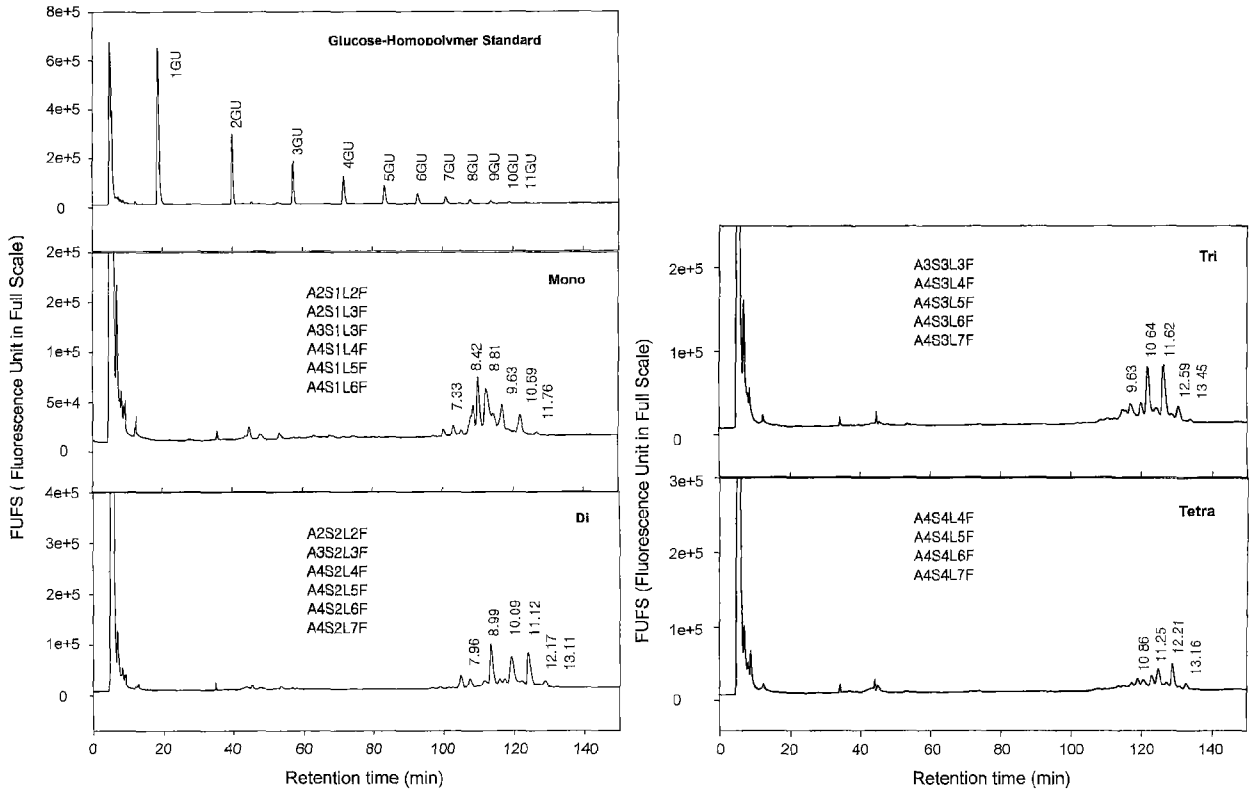

Fig. 3-1. GlycosepN chromatogram of control sample collected from MonoQ profile (Fig. 2) and determination of GU (Glucose Unit) value of peaks.

(tetra-) to 8.72% (tri-) and 1.25% (tetra-), respectively. The relative amount of disialylated glycan was hardly changed, yet the level of monosialylated glycan increased.

The fractions of sialylated oligosaccharides from the MonoQ were collected, aspirated, and freeze-dried. A glucose-homopolymer standard was injected into the

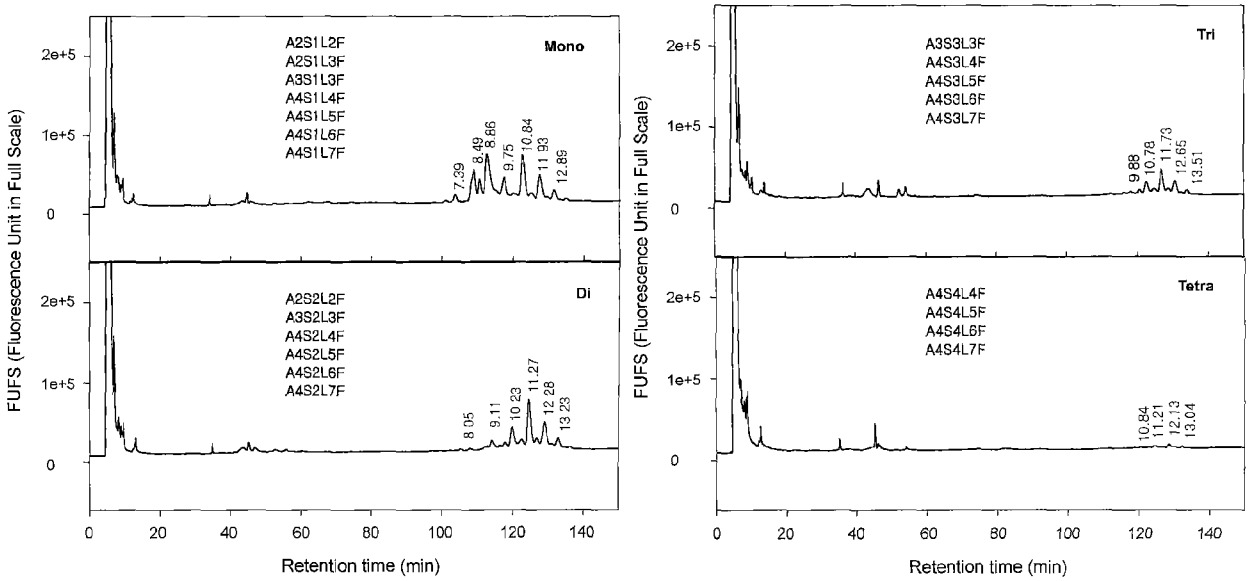

Fig. 3-2. GlycosepN chromatogram of NaBu-treated sample collected from MonoQ profile (Fig. 2) and determination of GU (Glucose Unit) value of peaks.

Glycosep-N column to set the standard GU (glucose unit) value before injecting the samples (Fig. 3-1). Based on the corresponding relationship between the GU value and the retention time of the glucose-homopolymer standard chromatogram, a multiparameter function was deduced, which was then used to calculate the GU value of the sample peaks by applying their retention time. Next, the fractions collected from the MonoQ were injected to Glycosep-N column and the GU values were calculated relative to the retention time. (Fig. 3) The structure of each oligosaccharide was predicted according to its GU value and the data previously reported by Guile *et al.* [21]. The oligosaccharide structures and relative amounts of each structure are summarized in Table 2. The nomenclature used to describe oligosaccharide structures was a modified version of the method used by Guile *et al.*: A(1-4) indicates the number of antennae linked to the trimannosyl core; S(1-4), sialic acid; L(1-7), the number of N-acetyllactosamine repeats; F, the core fucose (Fig. 4).

In the present work, it was found that the relative amounts of sialylated oligosaccharides were lower in the NaBu-treated sample than in the control. This result implies that the NaBu supplemented in the culture may have adversely affect the sialylation of EPO. According to

Table 2. Quantative amount of sialylated oligosaccharides according to their structure.

Oligosaccharide structure	GU value	Control (Relative amount, %)	NaBu-Treated (Relative amount, %)
A2S1L2F	7.36±0.03	0.86	1.15
A2S1L3F	8.46±0.03	5.34	3.26
A3S1L3F	8.84±0.02	10.12	13.24
A4S1L4F	9.69±0.06	4.68	3.90
A4S1L5F	10.77±0.07	2.06	7.91
A4S1L6F	11.85±0.08	0.31	4.78
A4S1L7F	12.89	ND*	1.56
A2S2L2F	8.01±0.04	1.14	0.29
A3S2L3F	9.05±0.06	4.73	3.10
A4S2L4F	10.16±0.07	5.56	5.77
A4S2L5F	11.20±0.07	4.91	11.17
A4S2L6F	12.23±0.05	0.85	7.61
A4S2L7F	13.17±0.06	0.09	2.14
A3S3L3F	9.76±0.12	2.44	0.48
A4S3L4F	10.71±0.07	6.17	1.42
A4S3L5F	11.68±0.05	5.47	2.88
A4S3L6F	12.62±0.03	1.18	1.64
A4S3L7F	13.48±0.03	0.15	0.37
A4S4L4F	10.85±0.01	1.82	0.11
A4S4L5F	11.23±0.02	2.77	0.28
A4S4L6F	12.17±0.04	3.11	0.46
A4S4L7F	13.10±0.06	0.59	0.28

*ND: Not Detected.

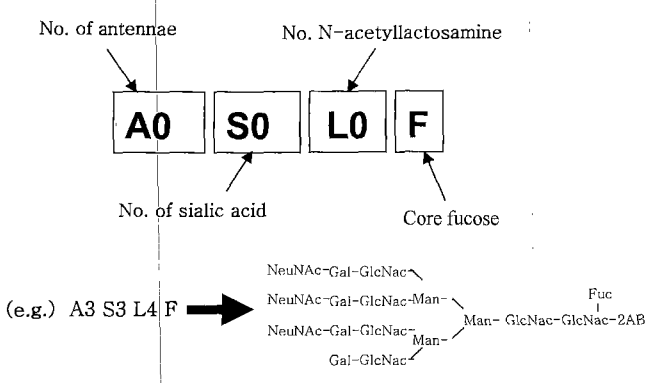

Fig. 4. Nomenclature for the description of oligosaccharide structures: A(1, 2, 3, 4) indicates the number of antennae linked to the trimannosyl core; L(1-7) indicates the number of N-acetyllactosamine repeats; F, core fucose.

the oligosaccharide structure anticipated by the Glycosep-N chromatogram, the core glycosylation did not appear to be affected by NaBu. However, the terminal silylation was the critical point. Sialytransferase transfers sialic acid into a terminal galactose residue. This reaction is known to occur in distal Golgi shortly before secretion, which is a very sensitive step. It can be completely and rapidly inhibited under a variety of conditions without a change in the secretion rate [22]. Accordingly, it would appear that the sialytransferase activity did not support the secretion rate of EPO when NaBu was supplemented. Consequently, under-sialylated EPO was released into the medium. This observation was similar to a previous report by Santell *et al.* [23]. They found that in 48 h after the addition of butyrate, the metabolically incorporated sialic acid rapidly and dramatically decreased.

This reduction in the contents of sialic acid under highly productive conditions can be overcome by amplifying the expression of the sialyltransferase gene. Weikert *et al.* [24] reported that human 2,3-sialyltransferase overexpression results in the sialylation of more than 90% of available branches.

Accordingly, to use NaBu for enhancing the production rate of glycoproteins, such as EPO, the overexpression of sialyltransferase is required, in which case, the production of high quality glycoproteins can be achieved.

Acknowledgment

This work was supported by a grant from Korea Science & Engineering Foundation.

REFERENCES

1. Jacobs, K., C. Shoemaker, R. Rudersdorf, S. D. Neill, and R. J. Kaufman. *et al.* 1985. Isolation and characterization of

genomic and cDNA clones of human erythropoietin. *Nature* **313:** 806–810.
2. Tsuda, E., M. Goto, A. Murakami, K. Akai, and M. Ueda, *et al.* 1988. Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins. *Biochemistry* **27:** 5646–5654.
3. Dordal, M. S., F. F. Wang, and E. Goldwasser. 1985. The role of carbohydrate in erythropoietin action. *Endocrinology* **116:** 2293–2299.
4. Takeuchi, M., N. Inoue, T. W. Strickland, M. Kubota, and M. Wada, *et al.* 1989. Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc Natl Acad Sci USA* **86:** 7819–7822.
5. Wasley, L. C., G. Timony, P. Murtha, J. Stoudemire, and A. J. Dorner, *et al.* 1991. The importance of N- and O-linked oligosaccharides for the biosynthesis and *in vitro* and *in vivo* biologic activities of erythropoietin. *Blood* **77:** 2624–2632.
6. Goldwasser, E., C. K. Kung, and J. Eliason. 1974. On the mechanism of erythropoietin-induced differentiation. 13. The role of sialic acid in erythropoietin action. *J. Biol. Chem.* **249:** 4202–4206.
7. Fukuda, M. N., H. Sasaki, L. Lopez, and M. Fukuda. 1989. Survival of recombinant erythropoietin in the circulation: The role of carbohydrates. *Blood* **73:** 84–89.
8. Spivak, J. L. and B. B. Hogans. 1989. The *in vivo* metabolism of recombinant human erythropoietin in the rat. *Blood* **73:** 90–99.
9. Dorner, A. J., L. C. Wasley, and R. J. Kaufman. 1989. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.* **264:** 20602–20607.
10. Oster, T., C. Thioudellet, I. Chevalot, C. Masson, M. Wellman, A. Marc, and G. Siest. 1993. Induction of recombinant human gamma-glutamyl transferase by sodium butyrate in transfected V79 and CHO Chinese hamster cells. *Biochem. Biophys. Res. Commun.* **28:** 406–412.
11. Palermo, D. P., M. E. DeGraaf, K. R. Marotti, E. Rehberg, and L. E. Post. 1991. Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression. *J. Biotechnol.* **19:** 35–48.
12. Oh, S. K. W., P. Vig, F. Chua, W. K. Teo, and M. G. S. Yap. 1993. Substantial overproduction of antibodies by applying osmotic pressure and sodium butyrate. *Biotechnol. Bioeng.* **42:** 601–610.
13. Chang, K. H., T. Endo, and J. H. Kim. 2000. Quantitative analysis of oligosaccharide structure of glycoprotein. *Biotechnol. Bioprocess Eng.* **5:** 1–6.
14. Bigge, J. C., T. P. Patel, J. A. Bruce, P. N. Goulding, S. M. Charles, and R. B. Parekh. 1995. Nonselective and efficient fluorescent labeling of glycans using 2- amino benzamide and anthranilic acid. *Anal. Biochem.* **230:** 229–238.
15. Lobb, R. R., J. W. Harper, and J. W. Fett. 1986. Purification of heparin-binding growth factors. *Anal. Biochem.* **154:** 1–14.
16. Najjam, S., B. Mulloy, J. Theze, M. Gordon, R. Gibbs, and C. C. Rider. 1998. Further characterization of the binding of human recombinant interleukin 2 to heparin and identification of putative binding sites. *Glycobiology* **8:** 509–516.
17. Sadir, R., E. Forest, and H. Lortat-Jacob. 1998. The heparan sulfate binding sequence of interferon-gamma increased the on rate of the interferon-gamma-interferon-gamma receptor complex formation. *J. Biol. Chem.* **273:** 10919–10925.
18. Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. *Science* **223:** 1296–1299.
19. Conrad, E. H. 1998. pp. 183–195. *Heparin-binding proteins* Academic press. NY, U.S.A.
20. Kanazawa, K., K. Ashida, M. Itoh, H. Nagai, H. Sasaki, and M. Fukuda. 1999. Establishment of a method for mapping of N-linked oligosaccharides and its use to analyze industrially produced recombinant erythropoietin. *Biol. Pharm. Bull.* **22:** 339–346.
21. Guile, G. R., P. M. Rudd, D. R. Wing, S. B. Prime, and R. A. Dwek. 1996. A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal. Biochem.* **240:** 210–226.
22. Thorens, B. and P. Vassalli. 1986. Chloroquine and ammonium chloride prevent terminal glycosylation of immunoglobulins in plasma cells without affecting secretion. *Nature* **321:** 618–620.
23. Santell, L., T. Ryll, T. Etcheverry, M. Santoris, and G. Dutina, *et al.* 1999. Aberrant metabolic sialylation of recombinant proteins expressed in Chinese hamster ovary cells in high productivity cultures. *Biochem. Biophys. Res. Commun.* **258:** 132–137.
24. Weikert, S., D. Papac, J. Briggs, D. Cowfer, and S. Tom, *et al.* 1999. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. *Nat. Biotechnol.* **17:** 1116–1121.