COMMUNICATION TO THE EDITOR

The Effect of Dissolved Oxygen (DO) Concentration on the Glycosylation of Recombinant Protein Produced by the Insect Cell–Baculovirus Expression System

Fuming Zhang,[1,2] Mark A. Saarinen,[1] Laura J. Itle,[1] Sybil C. Lang,[1] David W. Murhammer,[1] Robert J. Linhardt[1,2]

[1]Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52242
[2]Department of Chemistry and Division of Medicinal and Natural Products Chemistry, University of Iowa, Iowa City, Iowa 52242; e-mail: robert-linhardt@uiowa.edu

Received 4 May 2001; accepted 17 August 2001

Abstract: The effect of dissolved oxygen concentration on human secreted alkaline phosphatase (SEAP) glycosylation by the insect cell–baculovirus expression system was investigated in a well-controlled bioreactor. Oligomannose-type N-linked glycans (i.e., Man2 to Man6 and Man3F) were present in SEAP produced by *Spodoptera frusiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines. The relative amounts of the most highly processed glycans (i.e., Man3F and Man2 in the SEAP from Sf-9 and Tn-5B1-4 cells, respectively) were significantly higher at 50% of air saturation than at either 10% or 190% of air saturation. That is, glycan processing was inhibited at both low and high dissolved oxygen concentrations. © 2002 John Wiley & Sons, Inc. *Biotechnol Bioeng* **77**: 219–224, 2002.
Keywords: human secreted alkaline phosphatase (SEAP); baculovirus expression vector system (BEVS); insect cells; protein glycosylation; dissolved oxygen (DO) concentration

INTRODUCTION

The baculovirus expression vector system (BEVS) has a number of advantages over mammalian expression systems, including its ease of use, safety, and potentially high expression levels. In addition, the host insect cells are capable of most post-translational modifications observed in mammalian cells (Luckow, 1995). The utility of the BEVS is limited, however, by the inability of host insect cells to perform complex glycosylation of recombinant proteins (Kulakosky et al., 1998). Most recombinant mammalian glycoproteins produced with the BEVS have only terminal mannose *N*-glycan structures (Luckow, 1995).

Significant interest currently is focused on the function of carbohydrates as recognition determinants in a variety of physiological and pathological processes and the influence of carbohydrates on protein antigenicity, structural folding, solubility, and stability (Geisow, 1992; Sharon, 1998). It is highly desirable to develop a bioprocess system that can express recombinant glycoproteins with authentic (i.e., human-like) glycosylation. The inability to perform complex glycosylation in the BEVS is apparently due to the absence or insufficient levels of the terminal glycosyltransferases in the host insect cells that are required to convert *N*-linked side chains to complex forms (Jarvis et al., 1998). Recent studies have demonstrated that metabolic engineering can be used to extend the glycoprotein processing capability of insect cells to produce foreign glycoproteins with glycans that more closely resemble those produced by higher eukaryotes (Hollister et al., 1998; Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001). These studies involved the incorporation of mammalian glycosyltransferases into the insect cell.

In mammalian cell culture, many conditions have been demonstrated to affect protein glycosylation, including nutrient depletion (e.g., glucose, glutamine, or dissolved oxygen), accumulation of byproducts (e.g., lactate and ammonium ions), pH, cell age or density, cell growth state, and shear forces (Anderson and Goochee, 1994; Kunkel et al., 2000; Yang and Butler, 2000). Joshi et al. (2000) reported that the baculovirus type, host cell type, and growth medium all have strong influence on

Correspondence to: R. J. Linhardt
Contract grant sponsors: National Science Foundation
Contract grant number: BES 97-25094.

DOI 10.1002/bit.10131

the glycosylation pathway in insect cells, resulting in significant alterations in structures and relative abundance of *N*-linked glycoforms. Donaldson et al. (1999) reported the effect of DO concentration on the glycosylation of a recombinant protein (SEAP) by Tn-5B1-4 cells in spinner flasks. No studies have been reported regarding the effect of dissolved oxygen (DO) concentration on protein glycosylation in insect cells using a well-controlled bioreactor system.

In this article, secreted alkaline phosphatase (SEAP) was expressed with the BEVS and used as a model protein for investigating *N*-linked glycosylation. Recombinant SEAP provides an excellent model for studying *N*-linked glycan processing because it contains only a single occupied *N*-linked glycosylation site and no occupied *O*-linked sites (Endo et al., 1988). Three different DO levels (10%, 50%, and 190% of air saturation) were used to study the effect of (DO) concentration on the glycosylation of SEAP in the *Spodopterafrugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines in a well-controlled 3L bioreactor.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell Line and Virus

The *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5Bl-4 (Tn-5B1-4) insect cell lines were obtained from Gibco-BRL (Grand Island, NY) and Invitrogen (San Diego, CA), respectively. Recombinant *Autographa californica* multiple nucleopolyhedrovirus expressing human secreted alkaline phosphatase (AcMNPV-SEAP) under control of the polyhedrin promoter was obtained from Dr. H. A. Wood (Boyce Thompson Institute for Plant Research, Ithaca, NY).

Cell Growth

The Sf-9 and Tn-5B1-4 cells were grown in Sf-900 II serum-free medium (SFM) (Gibco-BRL) and Express Five SFM (Gibco-BRL), respectively. Cells were initially maintained in tissue culture flasks at 27°C. These cells were used to seed Erlenmeyer flasks at a cell density of ~0.5×10^6 cells/mL. Further scaleup prior to SEAP production utilized these cells (when the density reached 3–5×10^6 cells/mL) to seed larger Erlenmeyer flasks (up to 2 L) at ~0.5×10^6 cells/mL. The shaker flask cultures were grown at 27°C and rotated at 125 rpm.

Cell density and viability were determined with a Coulter Counter (Model ZM) interfaced with a Coulter Channellyzer (Coulter Electronics, Hialeah, FL) and by the trypan blue exclusion method, respectively.

SEAP Expression

Bioreactor runs were conducted in a 3-L water-jacketed bioreactor (Applikon, Foster City, CA) equipped with oxygen and pH electrodes (Mettler-Toledo, Wilmington, MA). Oxygen concentration and pH were monitored and controlled using a control system developed using Lab View software (National Instruments, Austin, TX). Temperature was controlled at 27°C, agitation at 150 rpm, the head space environment was maintained constant by flushing with air to avoid CO_2 accumulation (Mitchell-Logean and Murhammer, 1997), and the pH was maintained between 6.2 to 6.3 by addition of 1*M* KOH or 1*M* HC1. The bioreactor (working volume: 1.5 L) was seeded at a density of ~0.5×10^6 cells/mL with cells from shaker flask culture. Cells were grown at a DO concentration of 50% of air saturation to a density of ~1.5×10^6 cells/mL, at which time they were infected with AcMNPV-SEAP at a multiplicity of infection of approximately 5. At 4 h post-infection, the concentration of DO was changed to its final value (i.e., 10%, 50%, or 190% of air saturation) by adjusting the flow rate of pure oxygen.

SEAP Purification

Extracellular SEAP was purified using an affinity matrix as described by Zhang et al. (2001). The purity and molecular weight of the SEAP protein were assessed by SDS-PAGE (12% gel) using a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hercules, CA) and silver staining.

N-Glycan Release and Purification

Before performing glycan analysis, SEAP was further purified using a Sephacryl S-200 HR column (45 cm × 1.5 cm i.d.) to remove low molecular weight, non-proteinceous contaminants that might interfere with glycan analysis as described previously (Wolff et al., 2001). PNGase F (Boehringer Mannheim, Indianapolis, IN) was then used to remove the *N*-glycans from the purified SEAP. The resulting *N*-glycans were then purified (Wolf et al., 2001).

Fluorescent Labeling of *N*-glycans

Glycan-AGA (monopotassium 7-amino-1,3-napthalenedisulfanic acid (AGA), Aldrich Milwaukee, WI) conjugates were prepared by reductive animation in the presence of sodium cyanoborohydride (Lee et al., 1991; Wolff et al., 1999). Excess AGA was removed using a Sephadex G-25 column (45 cm × 1.5 cm i.d.) with detection at 247 nm. The fluorescently labeled glycan fractions were pooled and freeze dried.

Oligosaccharide Standard Preparation

An oligomannose-type oligosaccharide, oligomannose 8 (Oxford Glyco Systems, Inc., Wakefield, MA) was fluorescently tagged with AGA as described above. Mannose units were cleaved from the non-reducing termini of the oligosacchride by controlled α-mannosidase (20 U/mL, Jack bean enzyme, Oxford Glyco Systems, Wakefield, MA) digestion in 10 m*M* sodium acetate buffer (pH 5.0) at 37°C as described previously (Wolff et al., 1999). Oligosaccharides containing 3 to 10 saccharide units (Manl-AGA to Man8-AGA) were detected in the mixture of the products. Fluorescently labeled chitobiose (Sigma, St. Louis, MO) was added to complete the oligosaccharide standard mixture.

Exoglycosidase Digestion

The AGA-tagged SEAP N-glycans were digested with α-fucosidase (50 U/mL, source: bovine kidney, Oxford Glyco Systems, Wakefield, MA) in 10 mM sodium acetate buffer (pH 5.0) at 37°C for 2 days. The digestion was terminated by boiling the sample for 5 min. The denatured protein from a α-fucosidase was removed by centrifugation (10,000 × g for 10 min). The sample was treated with Ultra-micro-spin column to desalt before capillary electrophoresis analysis.

Glycan Analysis

The labeled glycan was analyzed with a capillary electrophoresis (CE) system (Dionex, Sunnyvale, CA) at 25 kV by fluorescent detection (λ_{ex} of 250 nm and λ_{em} of 420 nm) as described previously (Wolff et al., 1999).

SEAP Assay

SEAP activity was assayed by following the release of p-nitrophenol from p-nitrophenyl phosphate as described previously (Saarinen et al., 1999).

RESULTS AND DISCUSSION

Effect of DO Concentration on the Death of Virally Infected Insect Cells

The viability of AcMNPV-SEAP infected Tn-5B1-4 cells decrease more rapidly at increasing DO concentrations (Fig. 1A). In contrast, the decreased in the viability of the Sf-9 cells following viral infection was not affected by the DO concentration. The rate of Sf-9 cell death at all of the DO concentrations (10%, 50%, and 190% of air saturation) is between the death rates of the Tn-5B1-4 cells at 50% and 190% of air saturation, i.e., the Sf-9 cells died faster than the Tn-5Bl-4 cells except at high DO concentrations.

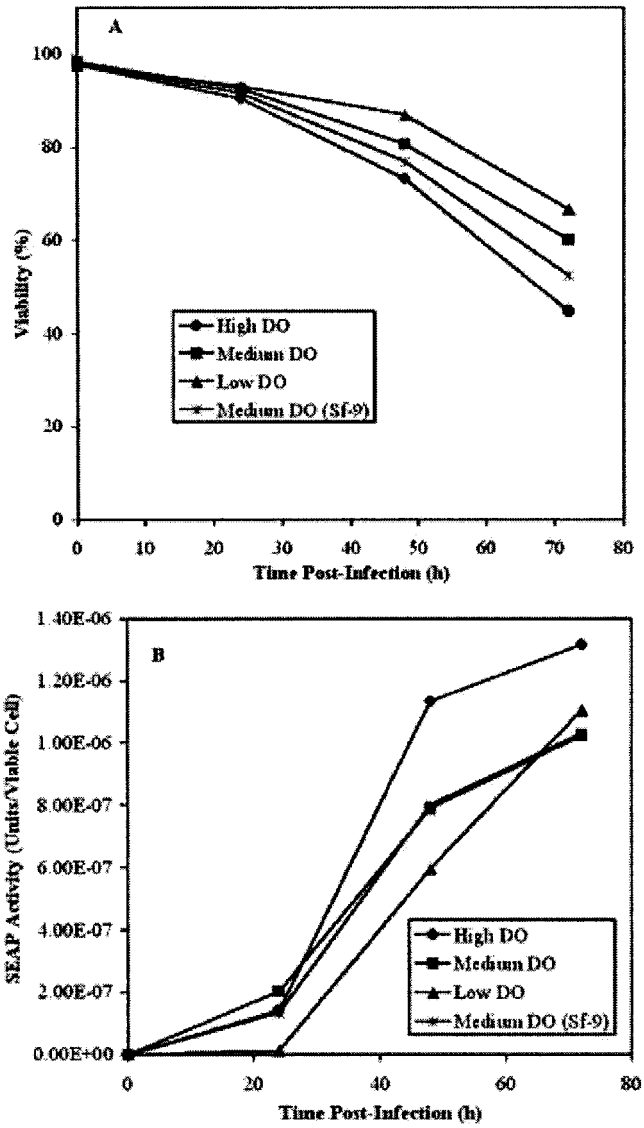

Figure 1. Behavior of AcMNPV-SEAP-Iinfected cells at various (DO) concentrations produced in a well-controlled 3L bioreactor. (**A**) Viability of Tn-5B1-4 cells as a function of time post-infection at low DO (10% of air saturation), medium DO (50% of air saturation), and high DO (190% of air saturation). In addition, the viability of the Sf-9 cells is given for medium DO concentration. Sf-9 cell viability for low and high DO concentrations follows closely that given for medium DO concentration. (**B**). SEAP expression levels (given in terms of units activity per viable cell at 0 h post-infection) of Tn-5Bl-4 cells as a function of time post-infection at low, medium, and high DO concentrations. In addition, SEAP expression levels of the Sf-9 cells are given for medium DO concentration. SEAP expression levels in the Sf-9 cells at low and high DO concentrations follow closely that given for medium DO concentration.

Effect of DO Concentration on SEAP Expression

The rate of SEAP expression (proportional to the slope) in the AcMNPV-SEAP infected Tn-5B1-4 cells increased with increasing DO concentration up to 48 h post-infection (Fig. 1B). By 72 h post-infection, SEAP accumulation at 10% and 50% of air saturation were essentially the same, whereas the accumulation at 190%

air saturation remained the highest. The rate of SEAP expression from 48 to 72 h post-infection, however, was highest at 10% air saturation. SEAP expression in the AcMNPV-SEAP infected Sf-9 cells did not vary appreciably with DO concentration. SEAP expression levels in the Sf-9 cells at all three DO concentrations were similar to the levels in the Tn-5B1-4 cells at 50% air saturation. These results are consistent with those found previously by Hensler et al. (1994), who reported no substantial influence of DO concentration on the final titer of (β-galactosidase produced by Sf-9 cells under a wide range of DO levels (5-100%) in 250 mL stirred reactors. Comparing the dynamics of the viability curves (Fig. 1A) and the corresponding SEAP expression curves (Fig. 1B) for Tn-5B1-4 cells suggests that an increase in DO concentration up-regulates the metabolism of the virally infected Tn-5B1-4 cell line, thereby leading to faster recombinant protein production and, ultimately, swifter cell death.

Effect of DO Concentration on *N*-glycan Processing in Insect Cells

The mixtures of purified, fluorescently-labeled *N*-glycans were characterized by CE mapping (Fig. 2, Table I). The corresponding glycan structures are given in Figure 3. Most of these peaks were identified through co-injection of glycan standards (data not shown). In addition, the fucosylated Man3 glycan (Man3F) peak was identified through the use of α-fucosidase, which converted this peak into the Man3 peak (data not shown). It was found that both the Tn-5B1-4 and Sf-9 cells produced oligomannose-type *N*-linked glycans (i.e., Man2 to Man6 and Man3F). Specifically, the Tn-5B1-4 cell line produced SEAP *N*-glycan structures ranging from Man2 to Man6, including Man3F. The major *N*-glycans produced in the Tn-5B1-4 cells were Man2 and Man3F. The Sf-9 cells produced Man3F and *N*-glycan structures ranging from Man4 to Man6, with Man3F and Man4 representing the major *N*-glycans.

The fraction of the most highly processed *N*-glycans (Man2 and Man3F in the Tn-5B1-4 and Sf-9 cells, respectively) were decreased at both 10% and 190% of air saturation compared to 50% of air saturation. Specifically, the ratio of Man2 *N*-glycan of SEAP was higher at 50% of air saturation (46% of total glycans) than at either 10% (37% of total glycans) or 190% of air saturation (32% of total glycans) in the Tn-5B1-4 cell line (Table I). Similarly, the ratio of Man3F *N*-glycan of SEAP was higher under 50% of air saturation (61% of

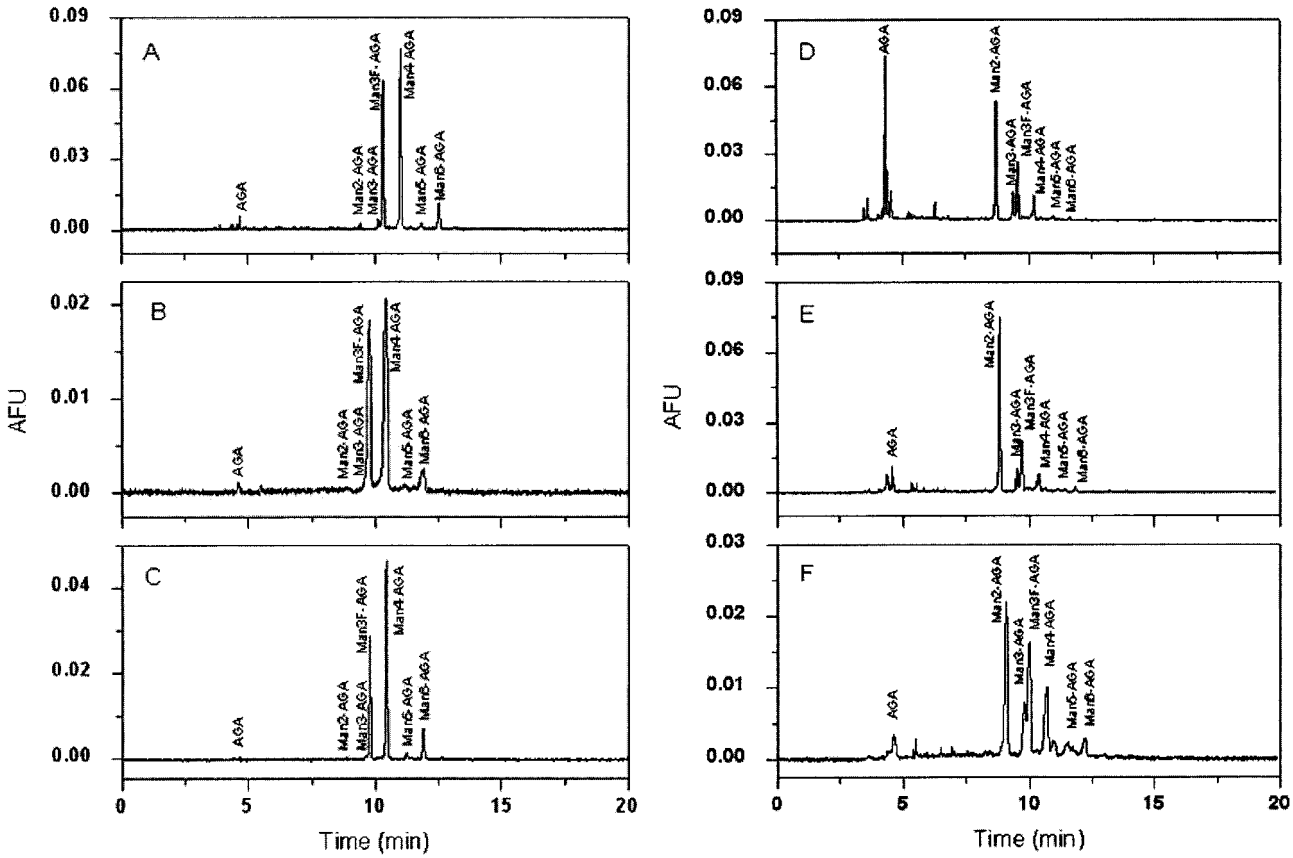

Figure 2. CE mapping of AGA-labeled *N*-glycans released from SEAP produced by Sf-9and Tn-5B1-4 cell lines at various DO concentrations in a well-controlled 3L bioreactor. (A) Sf-9 cell line, low DO;(B) Sf-9 cell line, medium DO; (C) Sf-9 cell line, high DO;(D) Tn-5B1-4 cell line, low DO;(E) Tn-5Bl-4 cell line, medium DO;(F) Tn-5Bl-4 cell line, high DO.

Table I. Summary of the ratio of *N*-glycans in-SEAP expressed by sf-9 and Tn-5 cell lines under different dissolved oxygen conditions (based on peak area obtained on capillary electrophoresis analysis.

	Sf-9			Tn-5B1-4		
	Low DO	Medium DO	High DO	Low DO	Medium DO	High DO
Man 2	1.2	ND*	ND	49	61	33
Man 3	1.3	ND	ND	11	9	11
Man 3F	37	46	32	24	22	29
Man 4	50	52	55	11	4.5	17
Man 5	1.2	0.6	1.6	2.3	1.8	5.2
Man 6	9.3	2.1	11	2.6	1.8	5.2

total glycans) than either 10% (49% of total glycans) or 190% of air saturation (33% of total glycans) in the Sf-9 cell line (Table I). Thus, these results demonstrate that the processing of SEAP *N*-glycans in both Sf-9 and Tn-5Bl-4 cells was inhibited at both low and high DO concentrations. Glycan analysis using CE shows low variability, and is capable of detecting a $< 10\%$ change in glycan level (Lee et al., 1991; Wolff et al., 1999; Choe et al., 2000; Wolff et al., 2001). The change observed with varying DO concentration in the most highly processed glycans is approximately 25%, suggesting that the changes are significant. The total distribution of different *N*-glycans in SEAP expressed by Sf-9 and Tn-5Bl-4 cell lines under different DO concentrations measured using CE are summarized in Table I.

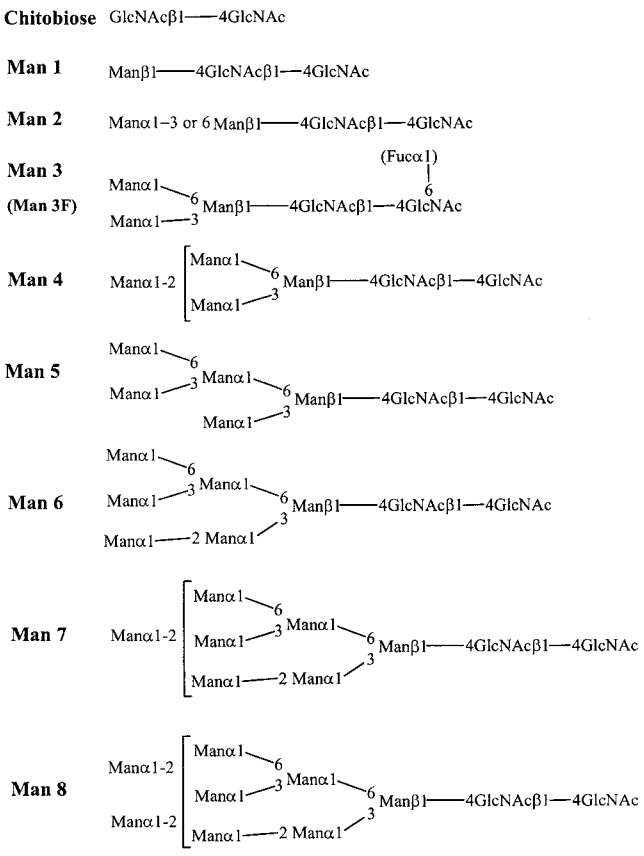

Figure 3. Chemical structure of *N*-glycan standards.

The *N*-glycan structures identified in the current study match well with the common structures produced in Tn-5Bl-4 cells reported by Donaldson et al. (1999). In contrast to our results, however, Donaldson et al. (1999) reported that varying the DO concentration did not affect oligosaccharide profiles of SEAP *N*-glycans produced in Tn-5Bl-4 cells in spinner flask studies. Specifically, they investigated the effects of low DO concentration (obtained by using a spinner flask with a tightly screwed cap) and high DO concentration (obtained by having the spinner flask open to an 80% oxygen atmosphere in the incubator). It should be noted that the DO concentration in the low DO spinner flask began at approximately 0% oxygen saturation at 0 h post-infection and increased to nearly 80% oxygen saturation (~400% air saturation) by 80 h post-infection. In addition, the high DO spinner flask was maintained at ~80% oxygen saturation throughout the infection cycle. The effect of DO concentration on the glycosylation of certain recombinant proteins has also been demonstrated in mammalian cell cultures. For example, in the production of human follicle-stimulating hormone from Chinese hanster ovary cells in perfusion culture, Chotigeat et al. (1994) reported that sialytransferase activity, sialic acid content, and specific productivity all increased when the DO concentration was increased from 10% to 90% of air saturation. In addition, Kunkel et al. (2000) reported that MAb produced in DO concentrations of 10%, 50% chinese hanste every, and 100% of air saturation in different bioreactors possessed the same types of biannary *N*-glycans, but the proportions of these oligosaccharide structures were strongly dependent on the DO concentration.

The authors are grateful for the AcMNPV-SEAP, provided by H. Alan Wood.

References

Anderson DC, Goochee CF. 1994. The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins. Curr Opin Biotechnol 5:546–549.

Breitbach K, Jarvis DL. 2001. Improved glycosylation of a foreign protein by Tn-5Bl-4 cells engineered to express mammalian glycosyltransferases. Biotechnol Bioeng 74:230–239.

Choe J, Zhang F, Wolff WM, Linhardt RJ, Murhammer DW, Dordick JS. 2000. Separation of AGP glycoforms using affinity-based reverse micellar extraction and separation. Biotechnol Bioeng 70:484–490.

Chotigeat W, Watanapokasin Y, Mahler S, Gray P. 1994. Role of environmental conditions on the expression levels, glycoform pattern and levels of sislytransferase for hFSH produced by recombinant CHO cells. Cytotechno 15:217–221.

Donaldson M, Wood HA, Kulakosky PC, Shuler ML. 1999. Glycosylation of a recombinant protein in the Tn5Bl-4 insect cell line: influence of ammonia, time of harvest, temperature and dissolved oxygen. Biotechnol Bioeng 63:255–262.

Endo T, Ohbayashi H, Hayashi Y, Ikehara Y, Kochibe N, Kobata A. 1988. Structural study on the carbohydrate moiety of human placental alkaline phosphatase. J Biochem 103:182–187.

Geisow MJ. 1992. Glycoprotein glycans roles and controls. Trends Biotechnol 10:333–335.

Hensler W, Singh V, Agathos SN. 1994. Sf-9 insect cell growth and β-galactosidase production in serum and serum-free media. Ann NY Acad Sci 745:149–166.

Hollister JR, Shaper JH, Jarvis DL. 1998. Stable expression of mammalian β 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. Glycobiology 8:473–480.

Hollister JR, Jarvis DL, 2001. Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mamalian β 1,4-galactosyltransferase and a 2,6-sialytransferase genes. Glycobiology 11:1–9.

Jarvis DL, Kawar ZS, Hollister JR. 1998. Engineering N-glycosylation pathways in the baculovirus-insect cell system. Curr Opin Biotechnol 9:528–533.

Joshi L, Davis TR, Mattu TS, Rudd PM, Dwelt RA, Shuler ML, Wood HA. 2000. Influence of baculovirus host cell interactions on complex N-linked glycosylation of a recombinant human protein. Biotechnol Prog 16:650–656.

Kulakosky PC, Shuler ML, Wood HA. 1998. N-glycosylation of a Baculovirus-expressed recombinant glycoprotein in three insect cell lines. In Vitro Cell Dev Biol-Animal 34:101–108.

Kunkel JP, Jan DCH, Butter M, Jamieson JC. 2000. Comparisons of the glycasylation of a monoclonal antibody produced under nominally identical cell culture conditions in two different bioreactors. Biotechnol Prog 16:264–270.

Lee KB, Al-Hakim A, Loganathan D, Linhardt RJ. 1991. A new method for sequencing linear oligosaccharides on gels using charged, fluorescent conjugates. Carbohydrate Research 214:155–168.

Luckow VA. 1995. Protein production and processing from baculovirus expression vectors. In: Shuler ML, Wood HA, Gradnador RK, Hanner HA, editors. Baculovirus expression systems and biopesticides. New York: Wiley-Liss. p 51–90.

Mitchell-Logean C, Murhammer DW. 1997. Bioreactor headspace purging reduces dissolved carbon dioxide accumulation in insect cell cultures and enhances cell growth. Biotechnol Prog 13:875–877.

Saarinen MA, Troutner KA, Gladden SG, Mitchell-Logean CM, Murhammer DW. 1999. Recombinant protein synthesis in Trichoplusia ni BTI-Tn-5Bl-4 insect cell aggregations. Biotechnol Bioeng 63:612–617.

Sharon N. 1998. Glycoproteins now and then: A personal account. Acta Anatomica 161:1–17.

Wolff MW, Murhammer DW, Linhardt RJ. 1999. Release and preparation of insect and unreducted N-linked oligosaccharides from Sf-9 insect cells. Prep Biochem & Biotechnol 29:1–21.

Wolff MW, Zhang F, Roberg JJ, Caldwell EEO, Kaul PR, Serrahn JN, Murhammer DW, Linhardt RJ, Weiler JM. 2001. Expression of Cl esterase inhibitor by the baculovirus expression vector system: preparation, purification, and characterization. Prot Express Purif 22: 414–421.

Yang M, Butler M. 2000. Effects of ammonia on CHO cell growth, erythropoietin production and glycosylation. Biotechnol Bioeng 68:370–380.

Zhang F, Wolff MW, Williams D, Busch K, Lang SC, Murhammer DW, Linhardt RJ. 2001. Affinity purification of secreted alkaline phosphatase produced by the baculovirus expression vector system. Appl Biochem Biotechnol 90:125–136.