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Enzyme Kinetics and Glycan Structural Characterization of Secreted Alkaline Phosphatase Prepared Using the Baculovirus Expression Vector System

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Abstract

Secreted human alkaline phosphatase (SEAP, a model protein containing a single *N*-glycan chain) was expressed in *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines infected with recombinant *Autographa californica* multiple nuclear polyhedrovirus expressing SEAP under control of the polyhedrin promoter. SDS-PAGE showed that both systems expressed fairly pure rSEAP products. The rSEAP expression level was 7.0 U/mL in Tn-5B1-4, higher than the 4.1 U/mL produced by Sf-9. Kinetic analysis showed that V_{max} and K_m of human placental SEAP were approx 10-fold higher than that of rSEAP, whereas the V_{max} and K_m of rSEAP prepared using both insect cell lines were comparable. To characterize the recombinant SEAP (rSEAP) glycosylation, the purified rSEAP was digested with PNGase F to release the *N*-glycan chains. Glycan analysis showed the presence of oligomannose-type *N*-linked glycans (i.e., $Man_{2-8}GlcNAc_2$ and $FucMan_{3\text{ or }4}GlcNAc_2$) in rSEAP from Sf9 and Tn-5B1-4 cell lines. The proportions of these oligosaccharide structures were different in the two cell lines. $Man_4GlcNAc_2$ and $FucMan_4GlcNAc_2$ were the major rSEAP *N*-glycans produced in Sf-9 cells, while $Man_2GlcNAc_2$ was the major rSEAP *N*-glycan produced in Tn-5B1-4 cells.

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Index Entries: Recombinant secreted human alkaline phosphatase (rSEAP); baculovirus expression vector system (BEVS); insect cells; enzyme kinetics; protein glycosylation.

Introduction

The baculovirus expression vector system (BEVS) has become well established for the production of recombinant glycoproteins. Its frequent use results from the relative ease and speed of which a heterologous protein can be expressed on the laboratory scale and the good chance of obtaining a biologically active protein *(1)*. The utility of the BEVS is limited, however, by the inability of host insect cells to perform complex glycosylation of recombinant proteins *(2,3)*. This inability to perform complex glycosylation is due to the absence or insufficient levels of the terminal glycosyltransferases required to convert *N*-linked glycan chains to complex forms *(4)*. 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 *(5,6)*.

Significant interest is currently 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 *(7,8)*. It is highly desirable to develop a bioprocesses system that can express recombinant glycoproteins with authentic (human-like) glycosylation. Secreted alkaline phosphatase (SEAP) provides an excellent model protein for investigating *N*-linked glycosylation because it contains only a single occupied *N*-linked glycosylation site and no occupied *O*-linked sites *(9)*.

In this article, recombinant secreted alkaline phosphatase (rSEAP) was expressed in *Spodoptera frugiperda* Sf-9 (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5B1-4) insect cell lines infected with recombinant *Autographa californica* multiple nuclear polyhedrovirus expressing SEAP under control of the polyhedrin promoter. The kinetic properties and *N*-glycan structure of the expressed rSEAP products were characterized and compared with human placental alkaline phosphatase.

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* Tn-5B1-4 (Tn-5B1-4) insect cell lines were obtained from Gibco-BRL (Grand Island, NY) and Invitrogen (San Diego, CA), respectively. Recombinant *Autographa*

californica multiple nuclear polyhedrovirus expressing human secreted alkaline phosphatase (AcMNPV-SEAP) under control of the polyhedrin promoter was obtained from Dr. H. A. Wood *(10)* (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 approx 0.5×10^6 cells/mL. Further scale-up prior to rSEAP production utilized these cells (when the density reached $3–5 \times 10^6$ cells/mL) to seed larger Erlenmeyer flasks at approx 0.5×10^6 cells/mL. The shaker flask cultures were grown at 27°C and rotated at 125 rpm.

Expression of SEAP with the Baculovirus Expression Vector System (BEVS)

Insect cells (400 mL in 2 L shaker flasks) cultivated under the same conditions as above were infected at a density of approx 1.0×10^6 cells/mL at multiplicity of infection (MOI) of 5.0. At 4 h postinfection (pi), cultures were centrifuged (500*g* for 5 min) to remove the old medium, and the infected cells were gently resuspended in an equal volume of fresh medium. The infected cultures were incubated in a shaking incubator (27°C, 125 rpm) for approx 72–86 h until the viability decreased to 70–80%.

Purification of rSEAP and Human Placental Alkaline Phosphatase

SDS-PAGE of rSEAP showed that the insect cell expressed products (approx 64 kD) were fairly pure with some low-molecular-weight contaminants (approx 10–20 kD). The purification of rSEAP was achieved by size exclusion chromatography using Sephadex G75 (Sigma). Human placental alkaline phosphatase was purified by affinity chromatography with the ligand 4-aminobenzyl-phosphonate, which is linked to histidyl-expoxy-activated-Sepharose *(11)*.

N-Glycan Release by N-Glycosidase F Digestion

Before performing glycan analysis, rSEAP was further purified using a Sephacryl S-200 HR column (45 cm × 1.5 cm id) to remove low-molecular-weight nonprotein contaminants that might interfere with glycan analysis. rSEAP was dissolved in column buffer (2 m*M* HEPES, 0.2% SDS, pH 7.5), loaded on the column, and then eluted in the void volume fractions. These fractions were pooled and the rSEAP was precipitated by adding nine volumes of 100% acetone, incubated overnight at –20°C, and the precipitated protein was recovered by centrifugation. The acetone precipitated rSEAP was resuspended in 0.2 mL of buffer 1 (20 m*M* HEPES, 1% SDS, pH 8.2) and β-mercaptoethanol (20 m*M*) was added. The sample was boiled

for 5 min to dissolve the protein and then combined with 0.8 mL of buffer 2 (20 m*M* HEPES, 1.25% NP-40, pH 8.2) containing 50 m*M* EDTA. PNGase F (Boehringer Mannheim, Indianapolis, IN) (1.0 U/mL) was added to remove the *N*-glycan chain from rSEAP. The reaction was carried out overnight at 30°C and stopped by boiling for 3 min. The released *N*-glycan was recovered from the core protein by fractionation on the S-200 HR column (45 cm × 1.5 cm id). Protein eluting from the column at the void volume was monitored by measuring absorbance at 280 nm. The PNGase-F-treated protein was analyzed by SDS-PAGE to confirm its complete deglycosylation. The fractions containing released glycan eluting in the included volume were determined by reducing sugar assay *(12)*.

Glycan Purification

After the glycan fractions were pooled, 2% (v/v) of saturated KCl was added and the solution was incubated at 4°C overnight. The precipitated potassium-SDS salt was removed by centrifugation, and the glycans in the supernatant were passed over a 3 mL Biobead SM2 (Bio-Rad, Hercules, CA) column to remove the residual nonionic detergent. The column was washed with five column volumes of water. The pooled fractions were freeze-dried and then desalted on a Sephadex G-10 (Pharmacia, Sweden) column. Salt-free fractions containing glycan were pooled and freeze dried.

Fluorescent Labeling of Glycan

Glycan-AGA [monopotassium 7-amino-1,3-naphthalenedisulfanic acid (AGA), Aldrich Milwaukee, WI] conjugates were prepared by reductive amination in the presence of sodium cyanoborohydride *(13)*. The monopotassium salt of AGA (recrystallized from water) was dissolved in 15% acetic acid (5 mg/100 μL), the purified glycan was added and the mixture was incubated for 1 h at room temperature. Sodium cyanoborohydride (100 μL of 1.0 *M*) was added to the mixture and incubated for 12 h at 45°C. Excess AGA was removed using a Sephadex G-25 column (45 cm × 1.5 cm id) with detection at 247 nm. The fluorescently labeled glycan fractions were pooled and freeze dried.

Preparation of Oligosaccharide Standards

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 nonreducing termini of the oligosaccharide by controlled α-mannosidase (20 U/mL, Jack bean enzyme, Oxford Glyco Systems) digestion in 10 m*M* sodium acetate buffer (pH 5.0) at 37°C. The digestion was monitored by capillary electrophoresis (CE). To obtain a mixture of oligomannose standards, samples were removed from the digestion at 10 min, 20 min, 1 h, 2 h, 3 h, 5 h, and 24 h, and additional starting material was added at 2 h and 5 h. The digestion was stopped by boiling the sample for 5 min. The denatured

protein from α-mannosidase was removed by centrifugation (10,000*g* for 10 min). The salt in the digestion buffer can interfere with CE analysis and was removed with Ultra-Micro Spin Column (G-10) (AmiKa, Columbia, MD). Oligosaccharides containing 3–10 saccharide units (Man1-AGA to Man8-AGA) were detected in the mixture of the products. Fluorescently labeled chitobiose (Sigma) was added to complete the oligosaccharide standard mixture (Fig. 6A).

Exoglycosidase Digestions

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

Glycan Analysis with Capillary Electrophoresis CE

The labeled glycan was analyzed with a capillary electrophoresis system (Dionex, Sunnyvale, CA) at 25 kV by fluorescent detection (λ_{ex} of 250 nm and λ_{em} of 420 nm). Separation and analysis were carried out in a reversed polarity mode using a fused silica capillary tube (55 cm in length and 50 μm id). The separation buffer contained 20 m*M* sodium phosphate pH 3.5. The sample was pressure injected (5 s, 5 psi) resulting in an injection volume of 0.5 μL.

Analytical

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.

rSEAP activity was assayed by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate *(14)*. Sample solutions were prepared by mixing equal volumes of supernatant (diluted if needed) and buffer (2 *M* diethanolamine, 1 m*M* $MgCl_2$, and 20 m*M* L-homoarginine, pH 9.8). Two hundred microliters of each resulting sample were placed into a well of a 96-well plate. Substrate solution (20 μL, 120 m*M* *p*-nitrophenyl phosphate in buffer) (1 *M* diethanolamine, 0.5 m*M* $MgCl_2$, and 10 m*M* L-homoarginine, pH 9.8) was added to each sample well and the absorbance at 410 nm was measured at 1-min intervals for a period of 15 min using a Dynatech plate reader (Dynatech Laboratories Inc., Chantilly, VA). OD/min values were calculated from linear portions of the resulting curves. These results were then converted to U/mL by using a calibration curve prepared using an alkaline phosphatase standard (Boehringer Mannheim, Indianapolis, IN).

The purity and molecular weight of the rSEAP protein were assessed by SDS-PAGE (12% gel) using a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hercules, CA) and silver staining.

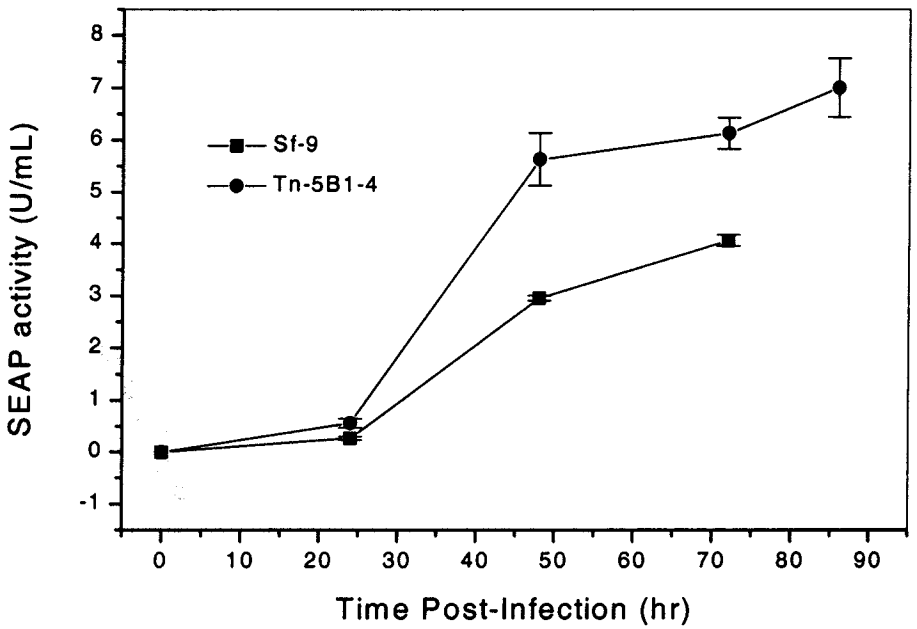

Fig. 1. rSEAP production in Sf-9 (filled squares) and Tn-5B1-4 (filled circles) cell lines in 2 liter shaker flasks.

Results

Recombinant SEAP Expression and Purification

Sf-9 and Tn-5B1-4 insect cells were infected with AcMNPV-SEAP at an MOI of 5 when the total cell density reached 1–1.5 × 10^6 cells/mL. The total cell density, cell viability, and rSEAP activity in both cell lines are shown as a function of time pi in Figs. 1 and 2. Following infection, cell density of the Sf-9 cell line increased approx 40%, while no change in cell density was observed for the Tn-5B1-4 cell line. The cell viability decreased from 99% at 0 h pi to approx 70% in the Sf-9 and Tn-5B1-4 cells, when the cultures were harvested at 72 h pi and 86 h pi, respectively. rSEAP activities reached 4.1 U/mL and 7.0 U/mL at the end of cultivation for the Sf-9 and Tn-5B1-4 cell lines, respectively. The different changes during pi in these two systems may be significant in terms of any heterogeneity of the expressed SEAP. SDS-PAGE (Fig. 3A) of the samples showed that rSEAP (MW approx 64 kD) was expressed in both cell lines and the products were fairly pure, i.e., in addition to the major rSEAP protein only some low-molecular-weight (approx 21 kD) protein contaminants were observed. The high-purity protein expression made the downstream purification easier. The rSEAP purification was achieved by size chromatography using a Sephadex G75 column. Pure rSEAP was obtained using this size exclusion chromatography (Fig. 3B). The apparent molecular weight of the rSEAP was approx 1 kD smaller than native SEAP purified from human placenta.

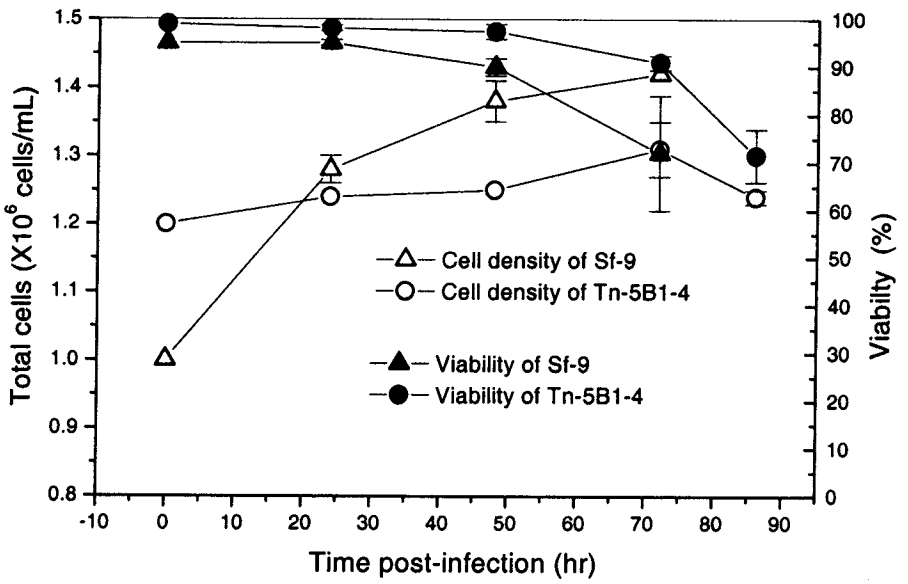

Fig. 2. Cell viability (filled symbol) and density (open symbol) change of infected cells in 2 liter shaker flasks. Sf-9 cell line (triangles); Tn-5B1-4 cell line (circles).

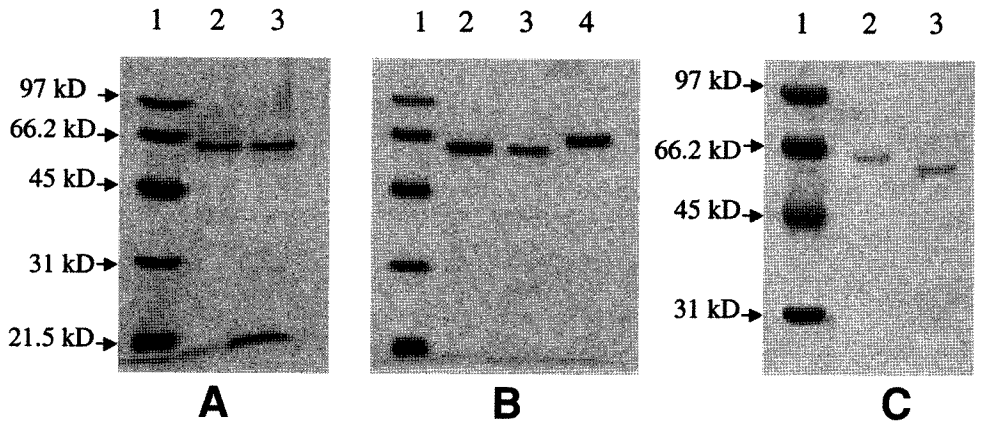

Fig. 3. SDS-PAGE analysis of rSEAP from the Sf-9 and Tn-5B1-4 cell lines infected with AcMNPV-SEAP. Panel A. Before purification: lane 1, markers; lane 2, rSEAP from Sf-9 cells; lane 3, rSEAP from Tn-5B1-4 cells. Panel B. After purification: lane 1, markers; lane 2, rSEAP from Sf-9 cells; lane 3, rSEAP from Tn-5B1-4 cells; lane 4, human placental alkaline phosphatase. The molecular weight of the markers in panel A and B are indicated at the left of panel A. Panel C. Sensitivity of rSEAP to PNGase F: lane 1, markers (molecular weights indicated at the left of panel); lane 2, purified rSEAP from Sf-9 cells; lane 3, purified rSEAP from Sf-9 cells treated with PNGase F.

This difference in apparent molecular weight was attributed to differences in the level of glycosylation of the proteins and the small difference in amino acid sequence.

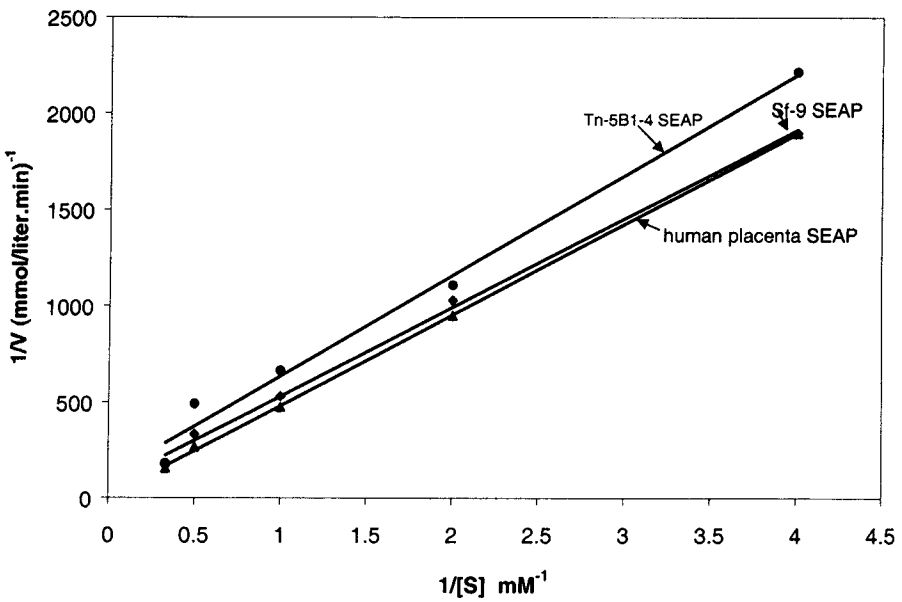

Fig. 4. The Lineweaver–Burk analysis of rSEAP and human placental alkaline phosphatase with *p*-nitrophenyl phosphate as substrate.

Table 1
V_{max} and K_m Values of rSEAP
from Sf-9, Tn-5B1-4 and Human Placental Alkaline Phosphatase

	Sf-9 rSEAP	Tn-5B1-4 rSEAP	Human placental alkaline phosphatase
V_{max} (mmol/L·min)	0.0146 (±0.004)	0.009 (±0.004)	0.1052 (±0.01)
K_m (m*M*)	6.76 (±1.7)	4.59 (±2.2)	49.7 (±6.3)

Enzyme Kinetic Properties

The enzymes were characterized to verify the functional activity of the rSEAP. Activity was measured for each enzyme with *p*-nitrophenyl phosphate substrate under the identical conditions. The specific activity of the purified Sf-9 rSEAP and Tn-5B1-4 rSEAP were 629 and 850 U/mg protein, respectively. These values were comparable to the specific activity of purified human placental alkaline phosphatase, 738 U/mg protein. Lineweaver–Burk analysis (Fig. 4) of rSEAP and human placental SEAP was used to calculate V_{max} and K_m (Table 1). The V_{max} and K_m of human placental SEAP are both considerably higher than the V_{max} and K_m of rSEAP prepared using Sf-9 or Tn-5B1-4 cell lines, whereas the V_{max} and K_m value for rSEAP prepared from both insect cell lines are comparable.

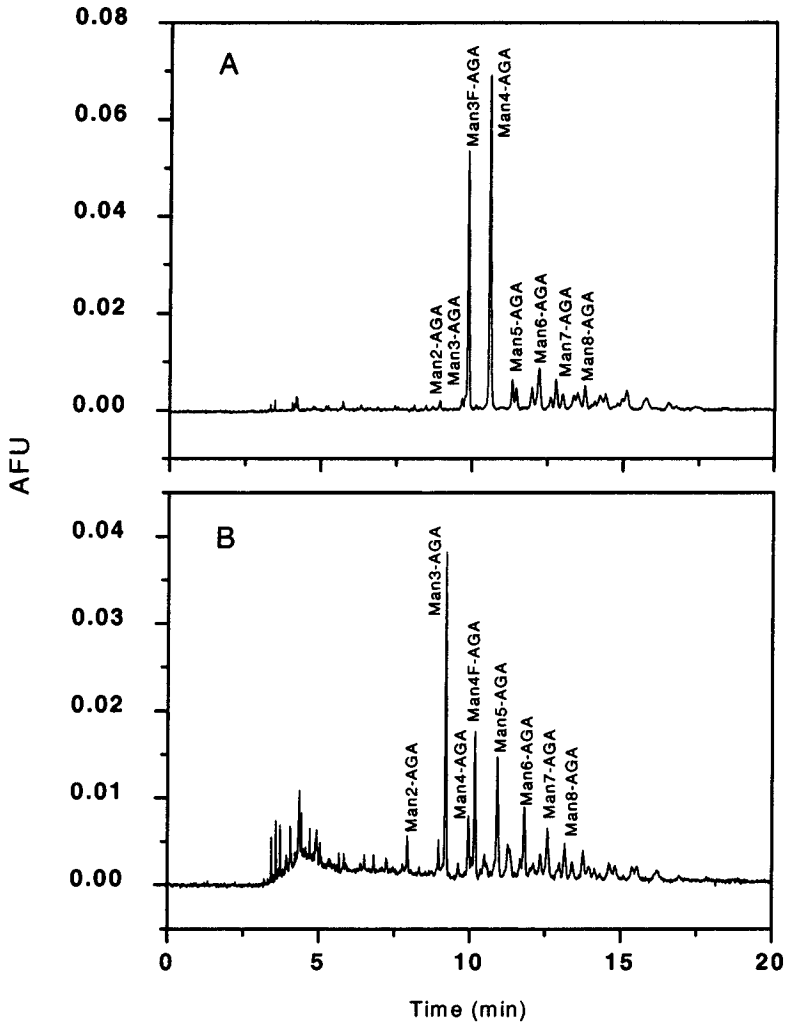

Fig. 5. CE mapping of AGA-labeled N-glycans released from rSEAP produced by Sf-9 and Tn-5B1-4 cell lines. **(A)** Sf-9 cell line, **(B)** Tn-5B1-4 cell line.

Analysis of Glycans with Capillary Electrophoresis

After *N*-glycans were released from the purified rSEAP by PNGase F digestion, the glycans were purified by removing SDS, nonionic detergent and then desalted. After PNGase F treatment, SDS-PAGE shows the complete disappearance of the band at 64 kD with the concomitant appearance of a new band at approx 62 kD (Fig. 3C), confirming that deglycosylation was complete. The resulting purified glycans were fluorescently labeled with AGA, an anionic fluorophore, so that any neutral oligosaccharides would be given a charge for migration on CE and to improve their detection *(15)*. Following tagging and the removal of excess AGA, oligosaccharide maps of the released glycans were obtained using CE (Fig. 5A and 5B). CE analysis with the Man 8 glycan standards (Fig. 6A)

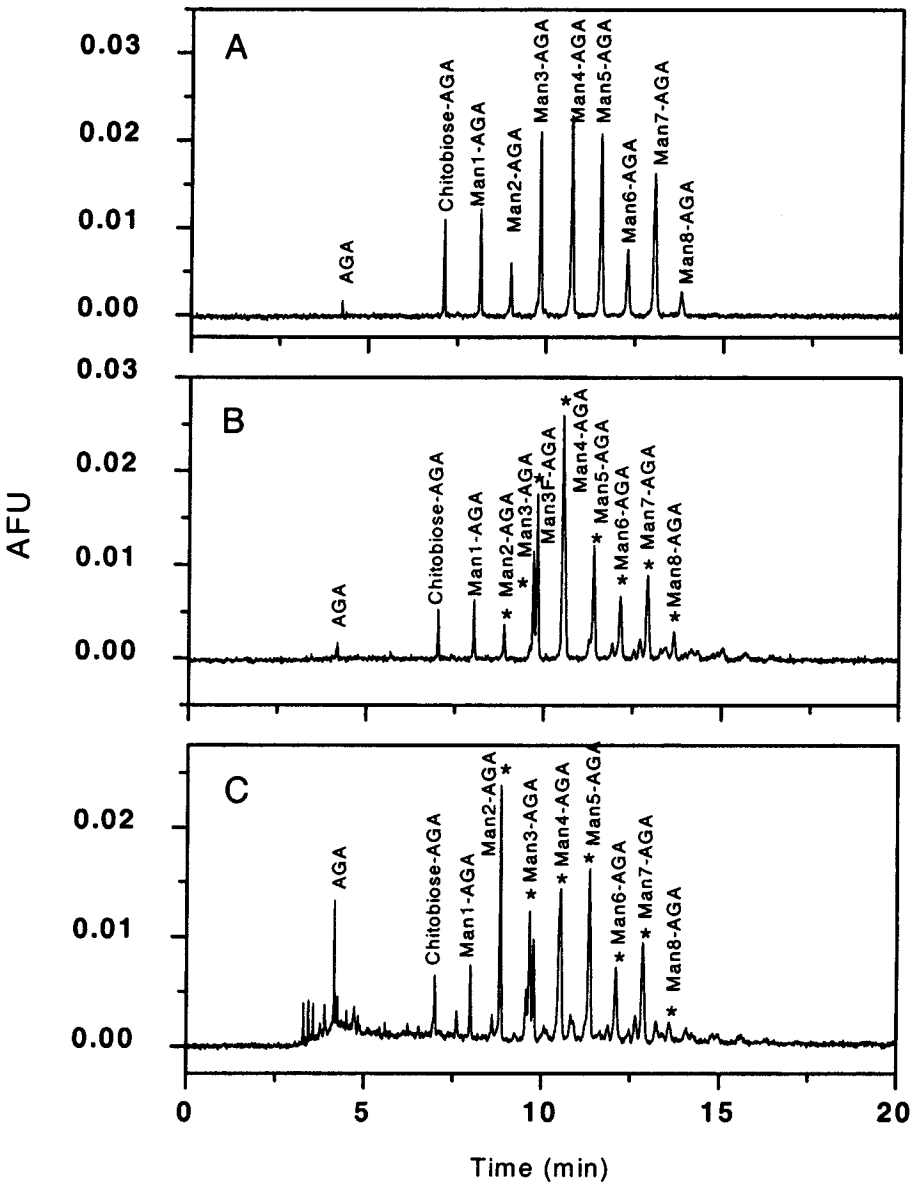

Fig. 6. CE analysis of Man 8 oligosaccharide standards and standards co-injected with rSEAP glycans. **(A)** Man 8 oligosaccharide standards; **(B)** Co-injection of the rSEAP glycans (from Sf-9 cell line) with Man 8 oligosaccharide standards; **(C)** Co-injection of the rSEAP glycans (from Tn-5B1-4 cell line) with Man 8 oligosaccharide standards. The asterisks in panels B and C indicate standards that co-migrated with glycans found in the Man8 oligosaccharide standard sample.

showed many of the same peaks were matching with the peaks observed in the maps of the *N*-glycans released from the rSEAP (see Fig. 7 for glycan structures). The coinjection of these standards with released *N*-glycans

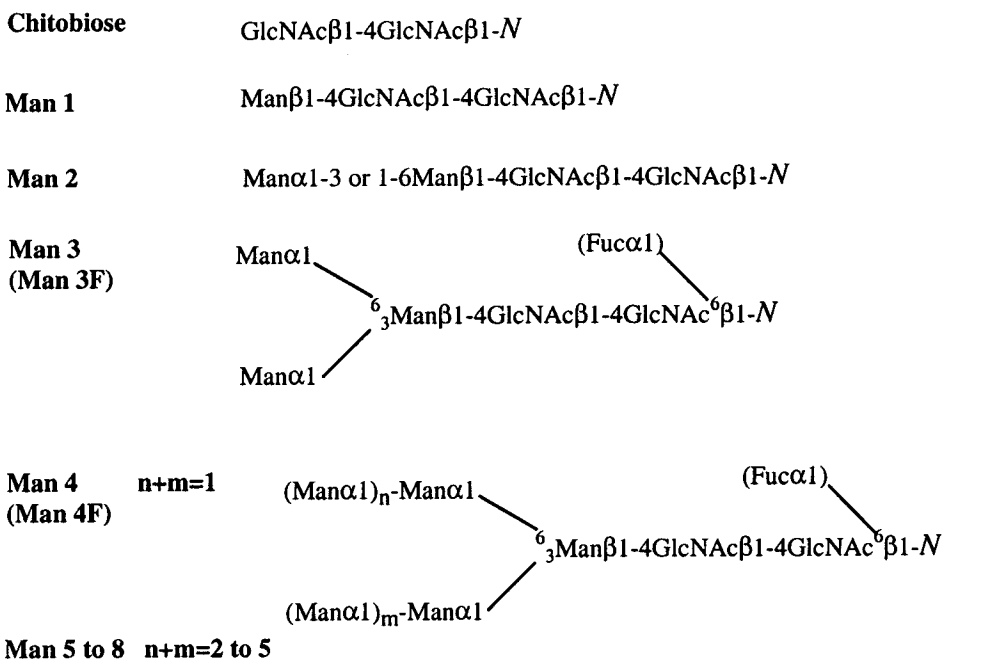

Fig. 7. Chemical structure of *N*-glycans: oligomannose- and fucosylated oligomannose-type *N*-linked glycans (Man2 to Man8 and Man3F, Man4F) were identified in rSEAP from Sf-9 and Tn-5B1-4 cell lines; complex-type biantennary structures found in human placental alkaline phosphatase *(9)*.

(Fig. 6B and 6C) showed that a mixture of oligomannose 2 to 8 glycans (Man2 to Man8) were present in the rSEAP obtained from Sf-9 and Tn-5B1-4 cell lines. Sensitivity to α-fucosidase digestion (data not shown) was used to identify fucosylated oligomannose 3 and 4 glycans (Man3F, Man4F) in the Sf-9 and Tn-5B1-4 rSEAP glycans, respectively (Figs. 5 and 7). The ratio of different glycans in rSEAP expressed by Sf9 and Tn-5B1-4 cell lines is summarized in Table 2, showing that the proportions of these glycan structures were dependent on the cell line. Man3F (30.2%) and Man4 (40.1%) are the major *N*-linked glycans in rSEAP obtained from Sf-9, whereas Man3 (25%) is the major *N*-linked glycan in rSEAP obtained from Tn-5B1-4.

Table 2
Ratio of *N*-Glycans in rSEAP Expressed by Sf9 and Tn-5B1-4 Cell Lines (Based on Peak Area Obtained from CE Analysis)

Glycan	Sf-9	Tn-5B1-4
Man2	1.5	2.4
Man3	1.7	25
Man3F	30.2	ND[a]
Man4	42.1	1.9
Man4F	ND[a]	14.7
Man5	3.0	12.0
Man6	5.2	7.3
Man7	2.2	3.8
Man8	2.9	3.8
Unidentified peaks	11.2	29.1

[a]ND, not detected.

Discussion

Insect cells infected with recombinant baculoviruses are useful for expressing recombinant proteins particularly when high production levels, secretion, and posttranslational modifications of the protein are desired *(2,16)*. There is special interest in the potential of insect cells to produce recombinant vertebrate glycoproteins with glycans similar to the original proteins, a feature lacking with many other expression vector systems *(3)*. The Sf-9 and Tn-5B1-4 cell lines used for rSEAP expression in this study are the most popular insect cell lines in BEVS. The rSEAP expression level (around 4–7 U/mL) is 10- to 100-fold higher than the concentration in placental tissue or in mammalian cell culture supernatant *(16)*. It is noteworthy that fairly pure rSEAP was expressed in both cell lines in the current study greatly simplifying the downstream processing.

Although a large number of recombinant glycoproteins have been produced with BEVS, the glycosylation of this expression system has not been studied systematically. Information available to date suggests that the processing of *N*-linked glycans on recombinant proteins produced in insect cells differs from that of the same protein produced in the vertebrate cells of their origin or in vertebrate cell culture expression systems *(17)*. Most recombinant mammalian glycoproteins produced with BEVS have only terminal mannose *N*-glycan structures *(2)*. In the current study, only oligomannose- and fucosylated oligomannose-type *N*-linked glycans (Man2 to Man8 and Man3F, Man4F, see Fig. 7) were identified in rSEAP from Sf-9 and Tn-5B1-4 cell lines. A previous study *(3)* showed that 19% of glycans from the Tn-5B1-4 cell line were core fucosylated, this is comparable to our result (14.7%) in the same cell line. Previous studies *(3,18)* relied on gel electrophoresis data and thus were of a qualitative nature. The glycan structures were determined quantitatively and for both Sf-9 and Tn-5B1-4

cell lines in the current study (Table 2). These glycan structures differ from the complex-type biantennary structures (Fig. 7) found in human placental alkaline phosphatase *(9)*. Glycosylation is known to affect the conformation, stability, immunogenicity, and biological activity of glycoproteins. Since SEAP has only one single glycan chain, we expected to observe little or no difference in the biological activity between the two rSEAPs and human placental alkaline phosphatase. Initial determination of enzyme activities ranged from 629 to 850 U/mg. A close examination of the kinetic properties of these enzymes revealed substantial differences between rSEAP and human placental alkaline phosphatase. It is an attractive theory to suppose that these differences are due to the differences in glycan structure, since the native human enzyme has complex sialylated structures, whereas those of the recombinant insect-produced form are of the truncated/fucosylated type. However, it should be borne in mind that of the 535 amino acids of the human pre-pro-phosphatase, certain residues were changed (2–5, 512, 514, 515, 518–522, 525–535) in order that the recombinant protein would have a secretion signal at the N-terminus, rather than the native C-terminal GPI additional signal *(19)*. Further studies will be directed at using insect cell lines that have been engineered to incorporate GlcNAcTI, GalT, and SiaT in order to obtain forms of recombinant phosphatases with human-like glycan structures. Evaluation of the activity and enzyme kinetics of these additional glycoforms will allow us to determine whether alterations in *N*-glycans are indeed the cause of such pronounced differences on the biological activity.

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References

1. Altmann, F., Staudacher, E., Wilson, I. B., and März, L. (1999), *Glycoconjugate J.* **16,** 109–123.
2. Luckow, V. A. (1995), Shuler, M. L., Wood, H. A., Granados, R. R., and Hammer, H. A. (eds.), Wiley-Liss, New York, pp. 51–90.
3. Kulakosky, P. C., Hughes, P. R., and Wood, H. A. (1998), *Glycobiology* **8,** 741–745.
4. Butters, T. D., Hughes, P. C., and Vischer, P. (1981), *Biochem. Biophys. Acta* **640,** 672–686.
5. Jarvis, D. L., Kawar, Z. S., and Hollister, J. R. (1998), *Curr. Opin. Biotechnol.* **9,** 528–533.
6. Hollister, J. R., Shaper, J. H., and Jarvis, D. L. (1998), *Glycobiology* **8,** 473–480.
7. Geisow, M. J. (1992), *Trends Biotechnol.* **10,** 333–335.
8. Sharon, N. (1998), *Acta Anatomica* **161,** 1–17.
9. Endo, T., Ohbayashi, H., Hayashi, Y., Ikehara, Y., Kochibe, N., and Kobata, A. (1988), *J. Biochem.* **103,** 182–187.
10. Davis, T. R., Trotter, K. M., Granados, R. R., and Wood, H. A. (1992), *Bio/Technol.* **10,** 1149–1150.
11. Zhang, F., Wolff, M. W., Williams, D., et al. (2001), *Appl. Biochem. Biotechnol.* **90,** 125–136.
12. Mopper, K. and Gindler, E. M. (1973), *Anal. Biochem.* **56,** 440–442.

13. Wolff, M. W., Murhammer, D. W., and Linhardt, R. J. (1999), *Prep. Biochem. Biotechnol.* **29(1),** 1–21.
14. Saarinen, M. A., Troutner, K. A., Gladden, S. G., Mitchell-Logean, C. M., and Murhammer, D. W. (1999), *Biotechnol. Bioeng.* **63,** 612–617.
15. Lee, K. B., Al-Hakim, A., Loganathan, D., and Linhardt, R. J. (1991), *Carbohydrate Res.* **214,** 155–168.
16. Heimo, H., Palmu, K., and Suominen, (1998), *Protein Express Purif* **12,** 85–92.
17. März, L., Altman, F., Staudacher, E., and Kubelka, V. (1995), in *Glycoproteins*, Montreuil, J., Viegenthart, J. F. G., and Schachter, H. (eds.), Elsevier, Amsterdam, pp. 543–563.
18. Kulakosky, P. C., Shuler, M. L., and Wood, H. A. (1998), *In Vitro Cell. Dev. Biol.–Animal* **34,** 101–108.
19. Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988), *Gene* **66,** 1–10.