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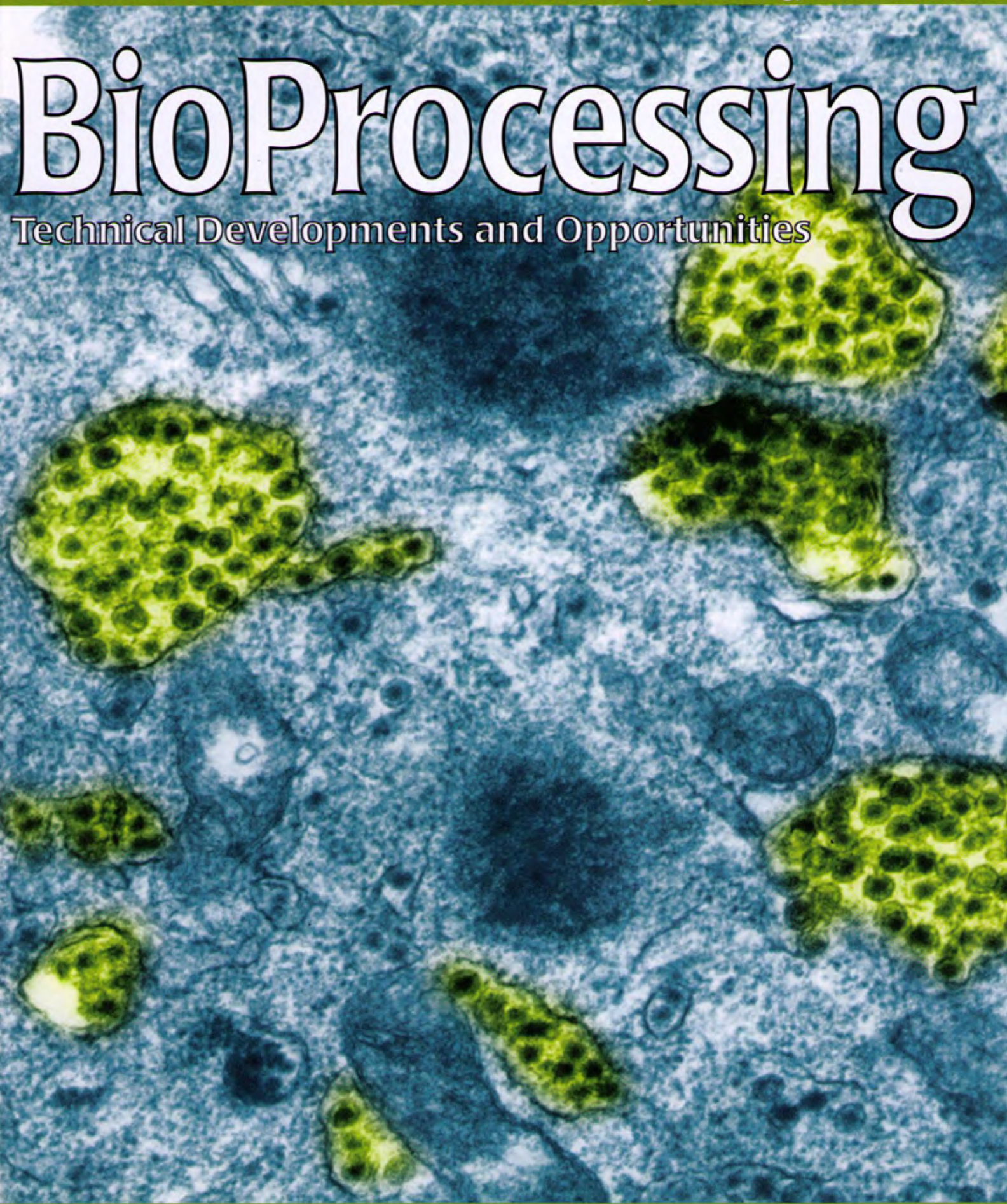

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Complex N-glycosylation of Recombinant Proteins by Insect Cells

BY LAURA A. PALOMARES
AND OCTAVIO T. RAMÍREZ

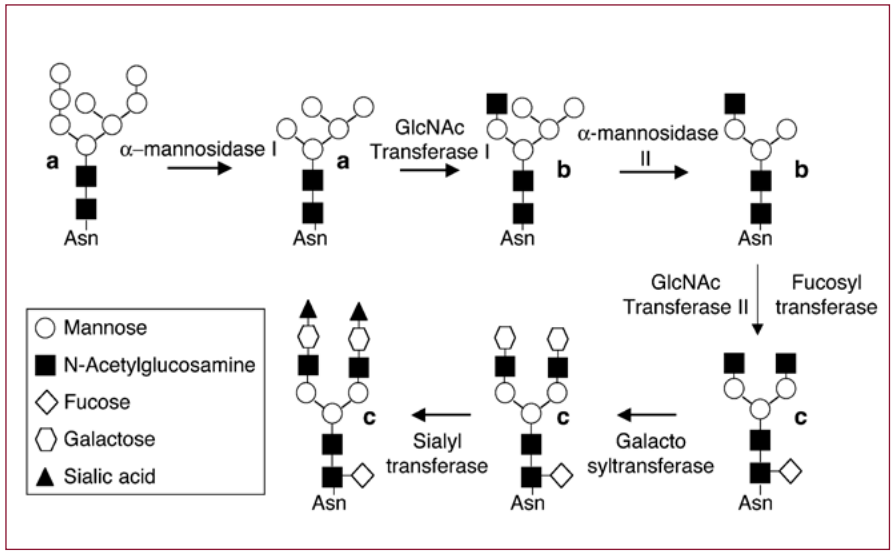

Figure 1. A possible route for glycan processing in the Golgi. a. High mannose forms. b. Hybrid forms. c. Complex forms.

The insect cell/baculovirus expression system typically results in more rapid expression and higher concentrations of recombinant proteins than what can be achieved with other animal cell culture systems. The lack of complex glycosylation in the proteins produced by this system, however, limits its use in the commercial-scale production of therapeutics. Complex glycosylation is required in many cases for adequate protein activity and pharmokinetic characteristics. In contrast to the protein's primary structure, which is encoded by the genetic material and is constant regardless of the host utilized, the extent of glycosylation is determined by the host, and by the protein itself. Even cells from different tissues of the same organism provide different glycosylation profiles. In addition, culture conditions and the cellular metabolic state can also influence protein glycosylation.[1]

In general, a host cell's ability to perform complex glycosylation is inversely related to protein yield. For example, bacteria can produce very high concentrations of recombinant proteins, but these proteins are usually improperly folded and lack most postranslational modifications, including glycosylation. In contrast, mammalian cells produce proteins with complex postranslational processing, but usually at very low concentrations and with low productivity. A system that produces high yields of a correctly processed protein would undoubtedly generate high levels of interest.

Protein N-glycosylation is a complex process that occurs in the cellular endoplasmic reticulum and Golgi apparatus, and it involves the sequential addition and removal of carbohydrates attached to an Aspargine of the protein. Modifications resulting in complex glycosylation occur in the Golgi apparatus. Figure 1 depicts a possible route of glycan processing in the Golgi. First, high mannose forms (with more than three mannose residues) are trimmed by mannosidases and transformed into hybrid (with one terminal residue different to mannose) and complex (no terminal mannose) glycans by the action of N-acetylglucosamine transferases (GlcNAcT). Further processing by galactosyltransferase (GalT) and sialic acid transferase (SialT) results in galactosylated and sialylated glycans, the

*Corresponding author **Dr. Laura A. Palomares** is a professor of biochemistry at the Instituto de Biotecnologfa, Universidad Nacional Autonoma de México; laura@ibt.unam.mx.*

forms most commonly found in mammalian proteins.[2]

Only high mannose and paucimannose (three or fewer mannose residues) glycans, which are not typical in mammalian proteins, have been found by most research groups in proteins produced by insect cells.[3] It should be noted that the majority of glycan analysis has been performed with low-sensitivity qualitative or semi-quantitative methods, such as lectin blotting or fluorophore-assisted carbohydrate electrophoresis. However, as more quantitative and sensitive methods (i.e. HPLC and mass spectrometry) are being utilized, more reports are appearing in which hybrid, complex, and even sialylated glycans are being found in the proteins produced by insect cells (Table 1). Cell lines from Pseudaletia unipuncta (A7S), Danaus plexippus (DpN1), Trichoplusia ni (Tn5B1-4 and Tn4h), Spodoptera frugiperda (Sf21), and Mamestra brassicae (MBO503) can produce a significant amount of complex glycans (5% - 63%). Interestingly, up to 33% of the glycans attached to proteins produced by insect cells have been reported to be sialylated (see Table 1). These results are controversial, as it has been believed that sialic acids were restricted only to deuterostomes.[4] Recent findings support the theory that insect cells can synthesize sialic acids, as reported by Kim, et al., who found a functional N-acetylneuraminic acid phosphate synthase gene in Drosophila melanogaster. This discovery confirms that at least one gene of the enzymatic machinery for sialic acid synthesis exists in insects, and that this machinery is similar to that found in humans.[5] In addition, sialic acids have been found in embryos of Drosophila melanogaster, larvae of Philaenus spumarius, and the testis of Galleria mellonella and Manduca sexta.[4,6,7,8] It can be concluded that some insect cells potentially have the required machinery for complex glycosylation, including sialylation, although the conditions required to consistently obtain such post-translational modifications are poorly understood. It is known that the synthesis of sialic acids by insects only occurs in some developmental stages.[4]

Even when complex glycosylation can be obtained from insect cells, high mannose and paucimannose forms can constitute up to 91% of the total glycans (Table 1). High mannose forms are the result of an incomplete processing in the Golgi, possibly caused by an oversaturation of the glycosylation machinery. This result can be prevented by decreasing the gene expression rate, or by expressing the gene in the early phase of infection. The process would involve using promoters that are weaker and/or earlier than the commonly utilized polh promoter, decreasing culture temperature, or reducing the multiplicity of infection.[9-17] On the other hand, paucimannose forms result from the action of an N-acetylglucosaminidase (GlcNAcase) that has been identified in insect cells.[18,19] As can be seen in Figure 2 (page 72), GlcNAcase cleaves the terminal GlcNAc residues from the glycan. Paucimannosidic glycans are no longer a substrate for GalT and constitute a dead end in glycan processing, while galactosylated glycans are not a substrate for GlcNAcase. The effect of the activity of each of these enzymes in the formation of complex glycans has been tested experimentally. Namely, GlcNAcase inhibition resulted in galactosylated and sialylated glycans in proteins produced by Tn5B1-4 cells.[20] In addition, the overexpression of GalT reduced the percentage of paucimannosidic forms and allowed the formation of galactosylated glycans (Table 1).[21]

Table 1. Some glycosylation profiles obtained from insect cells (adapted from 1 and 19).

Cell line	High mannose	Pauci-mannose[g]	Hybrid	Complex	Sialylated
Sf9[a]	ND	81%	19%	ND	ND
Sf9[c]	16%	84%	ND	ND	ND
Tn4h[c,f]	13%	86%	1%	ND	ND
Tn5B1-4[d]	31%	55%	14%	ND	ND
Tn5B1-4[c]	20%	71%	4%	5%	ND
A7S[c]	33%	55%	4%	8%	ND
DpN1[c]	24%	44%	6%	26%	13%
Tn5B1-4[b]	ND	35%	30%	35%	ND
Sf21[e]	50%	10%	ND	30%	30%
Ea4[a]	ND	22%	36%	42%	ND
Tn4h[c,f,h]	36%[i]		44%[j]		20%
Tn4h[c,f]	6%	43%	3%	48%	ND
MBO503[e]	35%	2%	ND	63%	33%

ND: not detected.

a. Profile of glycans attached to Asn25 of interferon γ.[40] b. Profile of glycans attached to IgG.[28] c. Profile of glycans attached to SeAP.[19,30,31] d. Profile of glycans attached to human transferrin .[21] e. Profile of glycans attached to human plasminogen.[29,41] f. Tn4h cells are derived from the Tn5B1-4 cell line. g. Forms with three or fewer mannose residues. h. Cultured under simulated low gravity (HARV reactor).[30] i. High mannose + paucimannose forms. j. Hybrid + complex

Both of these strategies (inhibiting GlcNAcase or increasing GalT activity) can be utilized to increase the concentration of complex forms. In addition to the action of GlcNAcase, it has been shown that the activities of GalT and SialT in insect cells are very low or absent.[19,22,23,24] Furthermore, baculovirus infection can affect the activity of glycosyltransferases.[19,25] Increasing the activity of both GalT and SialT should also result in higher amounts of complex glycans. The mechanisms that regulate the expression of both glucosyltransferases in insect cells are still unknown to date. Metabolic engineering has been utilized to increase the activities of both GalT and SialT by cloning mammalian genes into the baculovirus, or into stably transfected insect cells.[21,26,27] This approach has resulted in the detection of sialylated and galactosylated forms which could not be detected without the overexpression of the recombinant glucosyltransferases. It can be expected that a higher galactosyltransferase activity and a lower GlcNAcase activity will result in higher amounts of complex forms.

Another disadvantage of producing glycoproteins in insect cells is that their glycan core may be α1,3 fucosylated.[28,3] Fucosylation in such a position does not occur in mammalian proteins and is highly immunogenic.[3] Unfortunately, only the work of Hsu, et al. has quantified the percentage of α1,3 fucosylated forms in a recombinant IgG produced by Tn5B1-4 cells, and which ascended to 18% of total glycans.[28] While α1,3 fucosylation is definitely undesirable in proteins for therapeutic applications, it can be prevented by reducing the activity of the α1,3 fucosyltransferase, either by knocking out or repressing its gene, or by utilizing an inhibitor. To our knowledge, neither of these alternatives has been utilized.

Both culture conditions and the cellular metabolic state influence a complex process such as glycosylation, and culture mode is known to drastically influence glycosylation, also. Davidson and Castellino found that only cells in static culture perform complex glycosylation; and Joshi, et al. found that cells grown under simulated microgravity

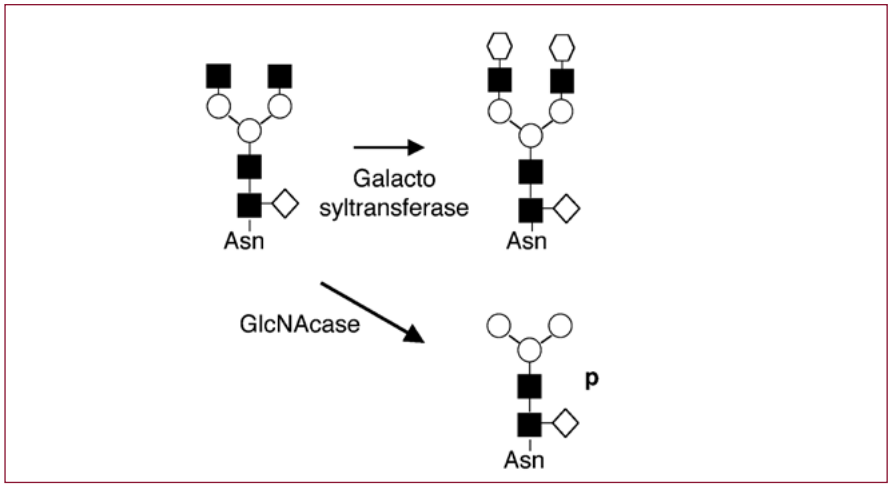

Figure 2. The action of GlcNAcase. p. Paucimannosidic forms.

sialylate.[29,30,31] Moreover, Zhang, et al. found that dissolved oxygen tensions (DOT) of 10% and 190% reduce the extent of glycosylation when compared to that measured in proteins produced at 50% DOT.[32] It can be expected that lower DOT, commonly found in non-controlled insect cell cultures, can further affect the extent of glycosylation.[33]

Medium composition also affects protein glycosylation.[31,34] Two groups have found that fetal bovine serum (FBS) is required to obtain sialylated glycans from insect cells.[31,35] FBS provides a wide variety of compounds which include growth factors, hormones, and lipids.[36] FBS also inactivates toxic materials, provides carrier proteins, contains protease inhibitors, affects the solubility of nutrients, has buffering capacity, protects cells from shear stress, and modifies some physical properties of culture medium such as surface tension and osmolarity.[36] It has been shown that FBS also reduces the rate at which baculovirus binds to cells.[37] It is unknown which effects of FBS, or its components, are responsible for improving protein glycosylation. FBS could contain precursors for the synthesis of sugar nucleotides required for glycosylation. Addition of precursors to culture medium results in more extensively glycosylated forms, as observed by Donaldson, et al. when mannosamine was added to cultures of Tn5B1-4 and Sf21 cells.[34] Additionally, when sialic acid phosphate synthase and CMP-sialic acid synthase are overexpressed in insect cells, they require N-acetylmannosamine feeding to obtain sialylated proteins.[38] In both reports, cultures are performed in serum-free media.

For glycosylation to occur, activated sugar nucleotides should be available. Tomiya, et al. quantified the concentration of sugar nucleotides in insect cells.[39] It was found that Sf9 and Tn5B1-4 cells contain sugar nucleotide pools that are more similar than those of mammalian CHO cells, except for CMP-sialic acid. This finding easily explains the lack of sialylated glycans often observed in proteins produced by insect cells, as well as the need for FBS components.[31,35] Moreover, synthesis of activated sugar nucleotides requires the sugar itself plus ATP. It can be anticipated that the stressful conditions that result in a reduced ATP pool may also result in a reduction of the sugar nucleotide pool.

Conclusion

Complex glycosylation and sialylation in proteins produced by insect cells require several factors. Very little is known about these factors and their mode of action, however, quantitative and systematic studies of glycosylation by insect cells in culture have started to appear. It can be expected that as the knowledge of insect cell glycobiology increases, more alternatives will emerge for obtaining a high productivity of

fully glycosylated proteins. We anticipate that both genetic and bioprocess engineers will publish research that greatly impacts the field.

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