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At present, several eukaryotic expression systems including yeast, insect and mammalian cells and plants are used for the production of recombinant proteins. Proteins with potential N-glycosylation sites are efficiently glycosylated when expressed in these systems. However, the ability of the eukaryotic expression systems to glycosylate may be not desirable for some proteins. If target proteins that do not carry N-linked glycans in the native host contain potential N-linked glycosylation sites, they can be aberrantly glycosylated in the eukaryotic expression systems, thus, potentially impairing biological activity. Recently, we have developed a strategy of enzymatic deglycosylation of proteins in vivo by co-introducing bacterial PNGase F via agroinfiltration followed by transient expression in plants. Here, we summarize our work on this topic and its potential implications.

The recent biotechnology boom has triggered an interest in using plants as an alternative platform for production of recombinant proteins. Plants offer several advantages compared with other recombinant protein expression systems, including simple, highly scalable, cost-effective manufacturing, product safety due to the lack of any harbored mammalian pathogens, and the presence of the eukaryotic post-translational modification (PTM) machinery. N-linked glycosylation is a PTM that is critical for correct folding, stability and biological activity of many proteins including recombinant subunit vaccines and therapeutic proteins produced in heterologous expression systems. However, some eukaryotic as well as bacterial proteins contain no N-glycans in the native host, but may contain multiple potential glycosylation sites which are aberrantly glycosylated when these proteins are expressed in heterologous eukaryotic expression systems, potentially leading to reduced functionality and immunogenicity due to incorrect or altered folding or masking of epitopes. For example, Pf48/45 protein of Plasmodium falciparum or A chain of human factor XIII do not carry N-linked glycans and protective antigen (PA) of Bacillus anthracis is not a glycoprotein; however, these proteins contain potential N-linked glycosylation sites which can be aberrantly glycylated during expression in yeast, mammalian or plant systems. It has been shown that aberrant N-glycosylation poses problems for many therapeutic applications. For example, aberrantly increased N-glycosylation is often observed in proteins of cancer cells. In addition, aberrant N-glycosylation of cell surface receptors, including integrins and cadherins, appears to be associated with changes in carcinoma progression and metastasis, indicating significant changes in these proteins’ behavior. In fact, the attachment of carbohydrates strongly affects physico-chemical properties of a protein, and therefore can alter its essential biological properties such as the specific activity, ligand-receptor interactions and immunogenicity, which may pose a safety risk when the protein is used in vivo. At this point, the ability of the eukaryotic expression systems to glycosylate may be not desirable for those targets that do not require N-linked glycosylation. Therefore, it is important to...
develop strategies for producing non-glycosylated forms of target proteins to preserve their native conformation and biological activity. One of such strategies is to use tunicamycin, a specific inhibitor of the enzyme that transfers acetylglucosaminephosphate (GlcNAc-1-P) onto dolichol phosphate (Dol-P) to block N-glycosylation. However, this approach has been previously demonstrated to result in a non-uniform expression of proteins in plants.14,15 Moreover, tunicamycin is very toxic, and even a short-term treatment of plants significantly affects protein folding,16,17 inhibits extracellular secretion of proteins,18 whereas a long-term treatment has a lethal effect on plants.19 Therefore, this strategy is not practical for production of recombinant proteins in a non-glycosylated form. Thus, we sought to develop a robust strategy to produce nonglycosylated protein targets that do not require N-glycosylation, similar to native Pfs48/45.

PNGase F is 34.8 kDa enzyme secreted by gram-negative bacterium Flavobacterium meningosepticum20 which cleaves a bond between the innermost GlcNAc and asparagine residues of high-mannose, hybrid and complex oligosaccharides in N-linked glycoproteins, except when the α(1–3) core is fucosylated. We hypothesized that co-expression of target protein with bacterial PNGase F in the endoplasmic reticulum may lead to its deglycosylation and allow for production of non-glycosylated forms of the protein in plants. Recombinant PNGase F has not been previously expressed in plants. Therefore, we first aimed to achieve the expression of active bacterial PNGase F in plants. The bacterial PNGase F sequence encompassing 314 amino acids (the full length of the catalytically active protein without a signal sequence) was optimized without a signal sequence) was optimized for expression in Nicotiana benthamiana plants, cloned into the plant expression vector pGRD44 and expressed in N. benthamiana plants with a FLAG tag. The expression of ~36 kDa PNGase F was confirmed by Western blot analysis using an anti-FLAG monoclonal antibody (mAb) (Fig. 1A). The average expression level of PNGase F was approximately 150 mg/kg of fresh leaf biomass. In plants, most proteins of the extracellular compartment and the endomembrane system are glycosylated and N-linked glycosylation of proteins has a great impact on their biological functions.21 In this regard, N. benthamiana plants expressing PNGase F remained healthy at 7, 8 and 9 days post inoculation (dpi) with no visible symptom development or change in growth when co-expression of target reached the highest level, suggesting that due to the transient nature of expression and brief time span, the effect of PNGase F on the endogenous protein folding and extracellular secretion is not significant. PNGase F was then purified from N. benthamiana leaves using an anti-FLAG agarose column, and its enzymatic deglycosylation activity was confirmed in vitro. Thus, since recombinant PNGase F has not been previously expressed in plants, our results support the utility of plants as an expression system for production of an active, endomembrane-free PNGase F at reduced costs.

To evaluate an in vivo activity of plant-produced PNGase F, the enzyme was transiently co-expressed in N. benthamiana with several recombinant proteins, including malaria vaccine candidate Pfs48F1, PA of B. anthracis and an antibody against PA of B. anthracis. Co-expression with PNGase F led to the accumulation of ~60 kDa Pfs48F1 protein in soluble fraction (Fig. 1B). The expression level of deglycosylated Pfs48F1 co-expressed with PNGase F was about 50 mg/kg of fresh leaf biomass and solubility was about 95%. Deglycosylated Pfs48F1 had a similar size as the in vitro deglycosylated Pfs48F1, suggesting that Pfs48F1 was enzymatically deglycosylated by PNGase F in vivo. The efficiency of Pfs48F1 in vivo deglycosylation was confirmed by the glycan detection and mass spectrometry analyses.1

Malaria is a vector-borne infectious disease caused by protozoan parasites. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa with over 300–500 million cases and more 1 million deaths each year from around the world. Although some vaccines are under development, no vaccine is currently available for this infectious disease that provides a high level of protection. Pfs48/45, a member of a Plasmodium-specific protein family.
III and V recognized the deglycosylated form of Pfs48F1 2–6-fold better than the glycosylated form of the same protein (Fig. 2). The binding affinity of mAb V for glycosylated Pfs48F1 and in vitro and in vivo deglycosylated Pfs48F1 was further evaluated using the KinExA instrument (Sapidyne) and was determined to be 9.7 nM, 3.8 nM and 2.7 nM, respectively. In addition, qualitative results of the signal inhibition analysis suggested that mAb III had the highest affinity to in vivo deglycosylated Pfs48F1 compared with both in vitro deglycosylated and glycosylated Pfs48F1. Taken together, these data suggest that aberrant glycosylation might have led to masking of important epitopes or caused incorrect/altered folding of Pfs48F1 and that deglycosylated Pfs48F1 may have a high TB activity and therefore, be a good candidate for malaria vaccine development.

Since the biological activity of many recombinant proteins depends on their glycosylation status, efforts are under way to humanize N-linked glycosylation and N-glycans of biopharmaceuticals produced in heterologous expression systems. Enzymatic deglycosylation of proteins in E. coli has been shown to be correctly folded (when co-expressed with 4 E. coli chaperones) and to elicit functional TB antibodies in mice.20,21 Pfs48/45 was also expressed in N. benthamiana plant at Fraunhofer USA Center for Molecular Biotechnology (FhCMB) using a transient expression system, but the TB activity of this plant-derived vaccine candidate was low. We hypothesized that the low TB activity of Pfs48/45 may be associated with an incorrect or altered folding or masking of important epitopes of the protein due to glycosylation. For example, a C-terminal fragment (containing 10 cysteine residues) of the Pfs48/45 protein of P. falciparum expressed in E. coli as a maltose-binding protein fusion has been shown to be correctly folded (when co-expressed with 4 E. coli chaperones) and to elicit functional TB antibodies in mice.20,21

Figure 2. Comparative ELISA analysis of glycosylated (●) and deglycosylated (■) forms of Pfs48F1. Recognition of glycosylated and deglycosylated forms of Pfs48F1 by various rat mAbs that detect epitopes I, IIb, III and V of Pfs48/45 was measured by ELISA.
vivo has not been achieved previously in any eukaryotic system, including plants. Our studies demonstrated that enzymatic deglycosylation of target proteins can be achieved in vivo by introducing a bacterial γ-D-glucosidase and recombinant protein in Nicotiana benthamiana plants by co-expressing bacterial PNGase F. Plant Biotechnol J 2012; 10:73-82. PMID:22052028.

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