Preliminary comparison of dextranase expression in two different host strains of the methylotrophic yeast *Pichia pastoris*

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The expression of dextranase encoding gene (*Dex*) into two different host strains of the methylotrophic yeast *Pichia pastoris* (MP36 and GS115) was compared. The *Dex* gene, encodes a mature protein of 574 amino acids and it was expressed using the *SUC2* signal sequence from *Saccharomyces cerevisiae* under the control of the *AOX1* promoter. In MP36 strain (*his3*$^-$) all transformants integrated the expression cassette and secreting an active enzyme to the medium after methanol induction. However, in GS115 strain (*his4*$^-$), only 44% of transformants expressed the active enzyme. We also found differences in the expression level between both strains, being in the MP36 expressing transformants higher than in the GS115 transformants.

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The food yeast *Candida utilis* as a host for heterologous gene expression: Construction of a vector system for high-level expression

Keiji Kondo

The yeast *Candida utilis* is generally recognized as safe and is allowed to be used as a food additive. The transformation of yeast has recently become possible, by using a drug resistance gene, which was constructed from *C. utilis* endogenous gene encoding ribosomal protein L41, as a marker. A vector system that facilitates high-level expression of a heterologous gene was also developed. The vector contains the strong promoter of the glyceraldehyde-3-phosphate decarboxylase gene from *C. utilis* and a promoter-deficient marker gene that allows high-copy-number integration of the vector. The vector has a unique structure that permits integration of the minimum sequences for gene expression and selection of the transformants at their target loci by single crossover recombination without the accompanying bacterial sequences. Application of this vector system to expression of a single-chain monellin gene encoding a plant sweet protein and a bacterial a-amylase gene resulted in extremely high-level expression, accounting for more than 50% of the total soluble protein. No significant decrease in the production level of both proteins was detected after 50 generations of non-selective growth, indicating that the yeast *C. utilis* is a promising host for protein production.

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Development of an integrative DNA transformation system for the yeast *Candida utilis*

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We report here the development of the yeast *Candida utilis* as a system for gene cloning and manipulation. To facilitate molecular studies in *C. utilis*, we isolated auxotrophic strains for uracil biosynthesis. A genomic library from the yeast *C. utilis* has been constructed and employed to isolate the *URA3* gene, encoding orotidine-5′-phosphate decarboxylase enzyme, by complementation in *Escherichia coli pyrF* and *Saccharomyces cerevisiae ura3* mutations. The deduced amino-acid sequence is highly similar to that of the Ura3 proteins from other yeasts. An extensive analysis of the family of orotidine-5′-phosphate decarboxylase is shown. Integrative plasmids were constructed based on the cloned *C. utilis URA3* gene and are applicable for directed insertions of genes of interest at the *URA3* locus through homologous recombination.

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Structural Analysis of Glycoproteins. Its Importance

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The meeting Biotecnología Habana'98 was held last November 16-21 at the Center for Genetic Engineering and Biotecnology in Havana, Cuba, focusing the attention of researchers of many countries all over the world on two fields: transgenesis and biotechnology applied to the industry. A general and exciting aspect was deeply discussed: the structural characterization of glycoproteins. Immediately, a question rises: why the interest in glycosylation analysis?

The answer could be: the importance of product glycosylation has led to a more detailed carbohydrate analysis since the beginning of the development of a new product. Improvements in glycosylation analysis have given scientists the possibility to judge how the glycan structures of recombinant glycoproteins compare to their natural human counterparts. Moreover, to keep the products' bioactivity or pharmacokinetics *in vivo* is in many cases a matter of maintaining

unaltered the specific glycan structures present in their natural form. However, to clearly understand the importance of glycosylation in biotechnologically produced glycoproteins, it is necessary to go inside the characteristics of this phenomenon.

The biosynthesis of protein-linked carbohydrate chains involves co- and post-translational events. This implies a genetic control over the structure of the final sugar chains produced, leading to the inherent heterogeneity of the glycosylation pattern. Consequently, a glycoprotein is generally composed of a discrete family of differently-glycosylated molecules called glycoforms, which have different physico-chemical and biochemical properties. This may, in turn, lead to functional diversity.

In many cases, it is not possible to directly associate a particular biological function with a specific carbohydrate structure. Structure-function relationships must be found in the context of the glycosylation pattern. Some are trivial physico-chemical effects that may, in turn, affect the biological function of the glycoprotein in a more complex way, for example, antigenicity, stability, half-life in plasma, intracellular routing, organ targeting, and cell-glycoprotein or cell-cell recognition event.

Glycosylation can be described as an event in which a considerable number of enzymes (glycosidases and glycosyltransferases) are involved as well as a process of an enormous energy requirement, then it is easy to understand that biotechnological glycoprotein production is influenced by several factors.

A biotechnological process consists of a synthesis and an isolation step. In the first one, the glycoprotein is obtained from producing cells. In the isolation phase, the glycoprotein is isolated and purified to "homogeneity". The synthesis and recovery procedures of the product are optimized. The final goal is to manipulate metabolic processes in genetically engineered strains to obtain a desired co- or post-translational modification of the product, to increase production rates and/or to reduce the formation of side-products. Hence, the initial choice of an expression system will continue to be of crucial importance.

The perspective to direct protein glycosylation by engineering strategies in order to obtain protein-linked carbohydrate chains, which are profitable for the pharmaceutical properties of the glycoprotein, is therefore attractive. For example, Gal-specific receptors on hepatocytes and macrophages do not recognize glycoproteins exhibiting their glycan moities capped with sialic acids. The masking of the β-Gal prevents early endocytosis, and hence reduces the clearance of the glycoprotein. This implies reduced clearance, which is correlated with the administration of lower doses of a therapeutic agent. Low doses are usually associated with fewer side effects. Furthermore, the development of glycosylation engineering strategies may facilitate elimination or masking of potential carbohydrate antigens avoiding undesirable immune responses. Glycosylation engineering deals with these aspects and shows that protein glycosylation can be influenced at each stage during a biotechnological process.

Glycosyltransferases and glycosidases, which are necessary for the biosynthesis of certain carbohydrate elements, are co-expressed with recombinant glycoproteins. For example, a heterologous rat α-2,6-sialyltransferase has been expressed in Chinese hamster ovary (CHO) cells. Whereas glycoproteins from CHO cells do not contain α-2,6-linked sialic acids, the α-2,6-sialyltransferase transfectoma CHO cells show the occurrence of surface glycoproteins containing α-2.6-linked sialic acid. The glycan moitey of a glycoprotein may be also remodeled *in vitro* using purified glycosyltransferases and/or glycosidases.

Summarizing, the objective of glycosylation engineering is to control the aspects influencing the glycosylation pattern of the product. Proteins unappropriately glycosylated may have undesired pharmacokinetics, possibly causing side effects during and after administration. The glycosylation engineering approach is therefore important to eliminate variations in the glycosylation pattern of a given glycoprotein within batch-to-batch productions, in such a way that the pharmaceutical properties of the molecule are not influenced negatively.

The number of biotechnologically produced glycoproteins approved for therapeutic use in humans is growing. Therefore, batch-to-batch consistency analysis of glycoprotein production, in terms of protein glycosylation, is needed. Currently, methods based on oligosaccharide profiling for batch control of glycoproteins are being developed. Identification of carbohydrate chains in oligosaccharide-mapping methods is commonly based upon the migration in electrophoresis and/or the retention time in chromatographic separation compared to standard compounds. Most of the mapping methods are restricted to neutral carbohydrate chains, so sialylated glycans have to be desialylated before mapping. In these approaches, the unraveling of native sialylation patterns is ignored. This type of mapping methodologies allows a fast comparison of batch-to-batch protein glycosylation. Moreover, when these methodologies are used in conjunction with lectin affinity and exoglycosidase digestion, the structural features of the oligosaccharides are described in detail. Many of these profiling methods are based on the fluorophore derivatization of the oligosaccharide population, which remarkably increases the sensitivity of the analysis.

Procedures using Mass Spectrometry as a detection system have overcome the problems associated with oligosaccharide-mapping methods where an incomplete characterization is achieved. Molecular masses and glycan fragmentation provide additional information about the structure of the carbohydrate chains. Several approaches including high performance liquid chromatography in on- or off-line configuration with Mass Spectrometry, have been described.

The aforementioned characteristics of the glycosylation process (which has persisted during evolution) were partially discussed in the symposium Structural Analysis of Glycoproteins during the last Biotecnología Habana'98 meeting, giving the attendees the possibility to be familiar with this complex but real problem when dealing with the production of genetically engineered proteins. Some of the outstanding lectures are included to facilitate the comprehension of this issue.

Mass spectrometric techniques for the rapid characterization of glycoproteins

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The complete characterization of the heterogeneous oligosaccharide population on a natural or recombinant glycoprotein is laborious and includes enzymatic or chemical liberation of the oligosaccharides, their chromatographic purification and final structural characterization of each fraction by NMR, MS and/or enzymatic methodes. Modern mass spectrometric techniques allow a substantial reduction of this workload. The direct mass spectrometric analysis of an intact glycoprotein yields information on the mass of the protein, as well as first hints on the heterogeneity of the glycan population present. After enzymatic or chemical liberation of the oligosaccharides, the analysis of the total native or derivatized glycan pool by MALDI/TOF- or ESI-MS separates all glycans present according to their molecular weight with higher sensitivity and better resolution than that of the best HPLC techniques. Additional structural information regarding the sequence of the monosaccharide residues, the presence of isomeric structures and, to a certain extent, valuable linkage information can be obtained by the MS/MS analysis of selected molecular ion species by collision induced decomposition from picomolar amounts of material. Thus, the terminal substitution pattern of complex type glycans typical for natural or recombinant glycoproteins like sialylated or non sialylated antennae, modification by peripheral or proximal fucose, the Gal 1-3Gal-R motif, lactosamine repeats, the Gal versus-GalNAc exchange and the presence of sulfate or phosphate groups can readily be determined from individual oligosaccharides of an unseparated glycan pool. Furthermore, parent ion scans can be used for the selective detection of the structural motifs mentioned above from very complex matrices. Complementary information about the glycosylation sites and the peptide backbone can be acquired by MALDI/TOF- or HPLC/ESI-MS analysis of the proteolytic peptide mixture obtained from the respective glycoprotein.

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Using a new profiling methodology for the characterization of N-glycosylation in natural and recombinant glycoproteins

José A Cremata, Raquel Montesino, Omar Quintero, Rossana García

Recently we described a simple and sensitive two-dimensional (2D) sugar-mapping technique of 8-amine-1,3,6-naphthalene trisulfonic acid derivatives (ANTS-derivatives) of neutral and sialyloligosaccharides for structure analysis and characterization of N-linked oligosaccharides using picomoles of samples. The method includes: 1) reductive amination with ANTS of enzymaticaly released oligosaccharides; 2) simultaneous separation of oligosaccharide derivatives in FACE and NH_2-HPLC column under ion suppression conditions; 3) plotting of the relative migration indexes (RMI) (X-axis) and relative retention times (t_r^{Man7}) (Y-axis) in graph; 4) when necessary, additional exoglycosidase digestion. The principal advantage of the new methodology here discussed is the finding of linear dependence within each group of compounds, i.e., oligomannosides, asialo complex oligosaccharides, sialylated diantennary, triantennary and tetraantennary structures.

The suitability of this methodology will be illustrated by the glycosylation profiling and structural analysis of oligosaccharide population of a series of glycoproteins of different origen which includes: recombinant proteins expressed in yeast, native fungal Endoglucanase 1, monoclonal IgG antibodies, and human a1 anti-trypsin.

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Glycosylation of cellobiohydrolase I from *Trichoderma reesei*

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By a combination of different techniques N-glycans from different strains of *Trichoderma reesei* have been analysed. The N-linked oligosaccharides have been released from CBH I using N-glycanase F and the complexity of the N-glycan mixture was evaluated by PAGE analysis after labeling the non reducing sugar ends with ANTS or AMAC. Biogel-P4 gelfiltration has been used to fractionate neutral oligosaccharides. These glycans were further analysed by HPAE-PAD chromatography and finally by NMR spectroscopy. With the RUTC 30 strain of *Trichoderma reesei* elucidation of certain N-glycan structures led to the conclusion that oligosaccharides were of limited sizes, that they were of the high-mannose type, and that substituent groups such as phosphates or glucoses were abundantly present. Characterizations of the N-glycan structures of *Trichoderma reesei* strain VTT D-80133 are in progress. PAGE and HPAEC.PAD analyses allready indicate that the latter strain synthesizes several different charged N-glycans (probably phosphorylated) not found with the RUTC 30 strain. A small amount of $Man_5GlcNAc_2$ was detected and NMR analysis gave proof that its structure was the same as that synthesized by mammalian cells. Indeed, this oligosaccharide was found to be acceptor substrate for human N-acetylglucosaminyltransferase I. With a third, uncharacterized, strain of *Trichoderma reesei*, the combination of PAGE and HPAEC.PAD analyses showed yet another N-glycan pattern on CBH I. Here, the presence of different glucosylated compounds could be suggested.

The conclusion is that fungal N-glycosylation shows strain dependent differences and that this can play a decisive role for future conversions of these glycans to a mammalian type.

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Deglycosilation of fungal enzymes for structure determination

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Although it is not the primary goal of our research, the problems we have encountered in the crystallisation of fungal cellulases, have forced us to acquire at least a rudimentary insight into how these enzymes are glycosylated. The glycosylation alters the surface properties of a protein and thereby how it may crystallise. Therefore heterogeneity, which is common in the carbohydrate portion, may have a detrimental effect. Some examples will be shown from studies of extracellular cellulose degrading enzymes from the filamentous fungus *Trichoderma reesei* (Tr herein) illustrating that glycosylation may vary not only between proteins, but also with the strain and the cultivation conditions. In the case of Cellobiohydrolase I (CBH1), which apparently requires a single GlcNAc at one site for crystallisation, it was possible to obtain a homogenuous preparation of the protein by choosing the right strain and proper cultivation conditions. The amount of sugar present on the catalytic domain was surprisingly small, only 3 single GlcNAc. The N-glycans are probably cleaved by an enzyme with similar specificity as Endo F and Endo H and the enzyme must be present in relatively large amounts in Trichoderma to cope with the high expression levels of CBH1. An easier way to obtain homogenuous protein preparations is to use enzymatic deglycosylation. We have developed a method by which we can deglycosylate several mg of protein for crystal structure determination at an affordable cost. A solution containing 10 mg protein and 0.2 mg Jack bean alpha-mannosidase (Boehringer-Mannheim) in 5 ml 0.1 M sodium acetate, pH 5.0, 2 mM zinc acetate, was sterile filtered and incubated overnight. Beta-mercaptoethanol and EDTA were aseptically added to 2 mM and 10 mM concentration, respectively, as well as 20 DGU (deglycosylation units) of a crude mixture of Endo F and PNGase F (Oxford Glycosystems). After overnight incubation, another portion of 20 DGU Endo F/PNGase F was added and the reaction left to proceed for two more days. All the incubations were done at 37 degrees C. We have applied this procedure on several of the fungal cellulases under study. Only in the case of the catalytic domain of Tr Endoglucanase 1 (EG1) we have made a proper characterization of the glycosylation before and after treatment. In the original preparation more than 10 glycoforms were present, the dominating one containing only a single GlcNAc and one Man. Some of the longer N-glycans were charged due to the presence of fosfate groups linked between the outermost mannose residues. After deglycosylation we could obtain a seemingly homogeneous preparation of the protein. Unfortunately we have not yet been able to obtain any crystals of the deglycosylated EG1. With the other enzymes the evaluation has been limited to wether the protein crystallises or not. Deglycosylation of CBH1 gave reproducible preparations that crystallised readily. Tr Cellobiohydrolase 2 (CBH2) on the other hand crystallises without deglycosylation although it has 2-3 N-glycans and 6 O-glycans attached and is heterogenuous. After deglycosylation, however, it does not crystallise under the same conditions. With CBH 58 from another fungus, Phanerochaete chrysosporium, it was only with the deglycosylated protein that we could obtain crystals and solve the structure. It should be remembered that this study only includes enzymes of fungal origin. Glycosylation is relatively simple, the only monosaccharides found being GlcNAc, Man and Glc. N-glycosylation is only of the high-mannose type and the O-glycosylation seems to be direct O-mannosylation and we havent seen any signs of the hyper-glycosylation commonly found in yeast. Although the method has proven useful for our studies, it may not work on proteins from other organisms and with more complex glycosylation.

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Bacterial 1,3-1,4-β-glucanases: mechanism, specificity and applications through functional redesign by protein engineering

Antoni Planas

The 1,3-1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73, 1,3-1,4-β-glucanases [1,2]) hydrolyze beta-glucans from the cell walls of Gramineae, which are particularly abundant in the starchy endosperm cells of cereals such as barley, sorghum, wheat and rice. 1,3-1,4-β-glucanase is an endoglycosidase acting with retention of configuration [3] and with a cleavage specificity for β-1,4 glycosidic bonds in 3-O-substituted glucopyranose units. The bacterial enzymes (family 16 of glycosyl hydrolases) are more active and thermally stable than the plant isozymes (family 17), and they are a target in our group [3-9] as an endoglycosidase model for studies on the mechanism of action and protein-carbohydrate molecular recognition, as well as for the redesign of the enzyme properties for biotechnological applications (brewing and animal foodstuff). This lecture summarizes recent studies focused on the details of the catalytic mechanism and the redesign of the enzymatic activity, and the structural/functional analysis of protein-carbohydrate interactions defining substrate specificity.

a) Catalytic mechanism. After identifying the essential catalytic residues by site-directed mutagenesis [4] (E138 as a general acid/base and E134 as a nucleophile in the *Bacillus licheniformis* enzyme), their functional role has been analyzed by a chemical rescue methodology on inactive mutants [4]. Addition of exogenous nucleophiles to the E134A and E138A mutants restores the enzymatic activity. Kinetic and structural analysis of the 'chemical rescue' indicates that the reactions proceed through different mechanisms and provides functional information on the catalytic pathway. The detection of a novel alfa-glycosyl formate intermediate will be discussed. It mimics the proposed glycosyl-enzyme intermediate in retaining glycosidases, and it is used to develop a new concept for rebuilding the active site towards new enzymatic activities.

b) Protein-carbohydrate interactions. 1,3-1,4-β-glucanases from *Bacillus* have an extended binding cleft containing 6 subsites, four on the non-reducing end

from the scissile glycosidic bond (-IV to -I) and two on the reducing end (+I and +II) [6]. By means of kinetic analyses with a series of oligosaccharide substrates with variable degrees of polymerization (obtained by chemical as well as enzymatic synthesis [7]), the contribution of each subsite to enzyme-substrate binding has been evaluated [8]. The amino acid residues involved in substrate binding belong to two well defined structural motifs: a surface loop that partially covers subsites -IV and -III, an a beta-sheet composed of 6 antiparallel beta-strands on the concave face of the protein molecule (jellyroll beta-sandwich structure as determined by X-ray crystallography of the free enzyme) [9]. Most of these residues have been mutated by site-directed mutagenesis. The structural/functional interpretation of protein-carbohydrate recognition based on the mutational analysis in combination with the molecular modeling will be discussed.

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Glycosylation profiling of high level heterologous proteins expression in the methylotrophic yeast *Pichia pastoris*

Raquel Montesino, Omar Quintero, Rossana García, José A Cremata

N-linked oligosaccharides from heterologous proteins secreted by the methylotrophic yeast *Pichia pastoris* under the control of alcohol oxidase (AOX1) promoter were characterized by a combination of Fluorophore-Assisted Carbohydrate Electrophoresis (FACE), using 8-amino-1,3,6-naphthalene trisulfonic acid (ANTS) as the fluorophore, and the separation of the same ANTS-oligosaccharide derivatives on NH_2-HPLC column. These proteins included: A bacterial enzyme, *Bacillus licheniformis* a-amylase; three fungal enzymes, *Saccharomyces cerevisiae* invertase, *Penicillium minioluteum* dextranase and *Mucor pusillus* aspartic protease; and two higher eukaryotic proteins, *Boophilus microplus* (tick) gut antigen and bovine enterokinase catalytic subunit.

$Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ (Man8, Man9) are the structures most frequently found in most of the characterized glycoproteins despite oligomannosides of lower degree of polymerization (Man6) were also found on invertase as well as higher structures (up to Man18) were common on aspartic protease and enterokinase. Phosphorylated oligosaccharides were observed on one protein, aspartic protease. In any case, oligosaccharides do not undergo terminal addition of a1,3-linked mannose. From these results, it is apparent that most foreign proteins secreted from *P. pastoris* are not subjected to the extensive mannosylation (hyperglycosylation) that commonly occurs to proteins secreted from *S. cerevisiae*. Additionally, slight changes in oligosaccharide profiles were observed when culture conditions were modified.

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The major exoglucanase secreted by *Saccharomyces cerevisiae* as a model to study protein glycosylation

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The major yeast exoglucanase (ExgIb) consists of a 408 aminoacid polypeptide carrying two short N-linked oligosaccharides attached to asparagines 165 (Asn165) and 325 (Asn325). These oligosaccharides are very similar, in both length and composition, to those present in the vacuolar protease carboxypeptidase Y. Minor glycoforms of exoglucanase arise by underglycosylation of the protein precursor (Exg165 and Exg325) or by elongation of the second oligosaccharide (ExgIa). The fact that these glycoforms can be readily separated and identified by HPLC and/or Western blots converts ExgI in an excellent model to study the role of the several components or branches of the precursor oligosaccharide in the eficiency and selectivity of the oligosaccharidyl transferase *in vivo*. We have found that the presence of a single glucose attached to Dol-PP-GlcGlcNAc2-Man9 increases the efficiency of transfer of that oligosaccharide to the protein acceptor. Also, the glucotriose unit appears to be involved in the selection of the sequons to be occupied, in such a way that its absence results in a bias towards the glycosylation of a particular sequon. Finally, we have shown the transfer of GlcNAc2 from Dol-PP-GlcNAc2 to exoglucanase, an indication that this intermediate is able to translocate from the cytoplasmic to the lumenal face of the endoplasmic reticulum membrane.

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