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Development of a *S. cerevisiae* whole cell biocatalyst for in vitro sialylation of oligosaccharides

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Abstract

Absence of sialylation on recombinant glycoproteins compromises their efficacy as therapeutic agents, as it results in rapid clearance from the human bloodstream. To circumvent this, several strategies are followed, including the implementation of a post-secretion glycosylation step. In this paper we describe the engineering of yeast cells expressing active surface exposed *Trypanosoma cruzi* trans-sialidase (TS) fused to the yeast Aga2 protein, and the use of this yeast in the sialylation of synthetic oligosaccharides. In an attempt to improve overall protein accessibility on the yeast surface, we abolished hyperglycosylation on the yeast cell wall proteins. This was achieved by disrupting the *OCH1* gene of the TS surface expressing strain, which resulted in increased enzymatic activity. Using a fluorescence-based activity assay and DSA-FACE structural analysis, we obtained almost complete conversion to a fully sialylated acceptor, whereas in the wild type situation this conversion was only partial. Increasing protein accessibility on the yeast surface by modifying the glycosylation content thus proved to be a valuable approach in increasing the cell wall associated activity of an immobilised enzyme, hence resulting in a more effective biocatalyst system.

Keywords: Trans-sialidase; Glycosylation; Yeast; Surface display; *Trypanosoma cruzi*

1. Introduction

Recombinant proteins offer great promise as therapeutics for a myriad of diseases. To obtain large quantities of these proteins, bacteria and lower eukaryotes are often used as expression hosts. The low cost of culturing the micro organisms, the potential high yield and the low contamination risk are just some advantages over mammalian expression systems. However, most proteins need post-translational modifications such as glycosylation in order to ensure long-term stability in human serum, and that is where the above-mentioned expression systems fail. Although the humanisation of the *N*-glycosylation pathway of lower eukaryotic expression hosts such as *Pichia pastoris* is in full progress (Vervecken et al., 2004; Hamilton et al., 2003;

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Bobrowicz et al., 2004), the resulting carbohydrate structures are still incomplete. In particular, they lack sialic acid, the terminal carbohydrate residue that protects the recombinant protein from fast clearance from the circulation by the hepatic asialoglycoprotein receptor (Lodish, 1991). This severely compromises the effect of a therapeutic glycoprotein in cases where long-term use is required (Chitlaru et al., 1998; Weigel, 1994).

Adapting the glycosylation pathway of expression hosts is just one way to obtain fully and correctly glycosylated end products. Another approach is the partial or full in vitro glycosylation of the recombinant protein after it has been secreted from the production cell, making use of soluble glycosyltransferases and their corresponding sugar donors to add missing sugars to the carbohydrate chain. Both *Saccharomyces cerevisiae* and *P. pastoris* have been successfully used to produce recombinant glycosyltransferases for this purpose (Herrmann et al., 1995; Borsig et al., 1997; Malissard et al., 2000). Use can also be made of glycosyltransferases that are immobilised on the yeast surface. As demonstrated recently by Jigami et al., intact yeast cells displaying glycosyltransferases on the surface can synthesise the desired oligosaccharide and function as a biocatalyst (Abe et al., 2003, 2004). A combination of yeast strains expressing different glycosyltransferases can lead to synthesis of complex oligosaccharides reminiscent of those present on natural glycoproteins.

Only two types of enzymes can transfer sialic acid to a growing sugar chain: the sialyltransferase family, the members of which are widely distributed across the animal kingdom (Harduin-Lepers et al., 1995), and the trans-sialidase (TS) family, the members of which are found only in some parasites, such as *Trypanosoma cruzi* (Schenkman et al., 1994), *Trypanosoma brucei brucei* (Engstler et al., 1993), *Trypanosoma congolense* (Engstler et al., 1995) and protozoan parasites such as *Endotrypanum* species (Medina-Acosta et al., 1994). The two enzymes have totally different properties. Unlike the typical sialyltransferases, which use an activated sialic acid molecule (CMP-Neu5Ac) as a monosaccharide donor (Paulson and Colley, 1989), the trans-sialidase catalyses transfer of preferably α-2,3-carbohydrate linked sialic acid from a donor to an acceptor carbohydrate, thereby forming a new α-2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine (Vandekerckhove et al., 1992; Ferrero-Garcia et al., 1993; Scudder et al., 1993). Buschiazzo et al. (2002) recently described the crystal structure of *T. cruzi* trans-sialidase, and showed that binding of sialyllactose induces a conformational switch that results in activation of the enzyme and modulation of acceptor binding.

In the present study we constructed yeast cells that display the *T. cruzi* trans-sialidase through the yeast surface display system (Boder and Wittrup, 1997). To achieve this, the trans-sialidase was C-terminally fused to the yeast Aga2 protein. Following Aga1–Aga2 heterodimerisation, the protein is targeted to the outer layers of the cell wall, where it becomes accessible for interaction with possible ligands. We show that the TS protein is correctly expressed and displayed on the yeast surface in fully active form. The intact yeast cells are able to perform sialylation of synthetic oligosaccharides, and can thus be regarded as candidate biocatalysts in the sialylation of non-sialylated and undersialylated heterologous proteins.

2. Materials and methods

2.1. General growth conditions

Yeast cells were grown overnight to saturation in 2% dextrose, 0.67% yeast nitrogen base and 1% casamino acids. The cells were diluted and grown for another 6 h to an optical density (OD_{600}) of 0.5. To induce surface expression of the recombinant protein, cells were pelleted by centrifugation, washed twice and resuspended to an OD_{600} of 0.5 in 2% galactose, 0.67% yeast nitrogen base and 1% casamino acids and incubated at 20 °C for 24–60 h.

2.2. Construction of the S. cerevisiae EBY100 OCH1 KO strain

The *S. cerevisiae OCH1* gene (GenBank accession no. D11095) was amplified from genomic DNA of *S. cerevisiae* EBY100 (Invitrogen, San Diego, CA) using the oligonucleotides 5′-CGAGCTCGATCATG-TCTAGGAAGTTG-3′ and 5′-GCTCTAGACTCGT-TATTTATGACCTGC-3′ as starter sequences. The PCR fragment was digested with *Sac*I and *Xba*I and ligated in the pSP64-vector (Melton et al., 1984) to give pSP64*OCH1*. The kanamycin resistance marker was obtained as a *Bam*HI/*Eco*RI (T4 DNA-polymerase