Vol. 210, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 5, 1995 Pages 14-20

SCALED-UP EXPRESSION OF HUMAN α2,6(N)SIALYLTRANSFERASE IN SACCHAROMYCES CEREVISIAE

Lubor BORSIG[1], Svetoslav X. IVANOV[1], Guido F. HERRMANN[2], Udo KRAGL[2], Christian WANDREY[2], and Eric G. BERGER[1*]

[1] Institute of Physiology, University of Zurich, CH-8057 Zürich, Switzerland

[2] Institute of Biotechnology 2, Forschungszentrum Jülich GmbH, D-52425-Jülich, Germany

Received March 27, 1995

Expression of recombinant full length human α2,6(*N*)sialyltransferase has been scaled-up in *S. cerevisiae* in a 150-l bioreactor yielding 47 U at a concentration of 0.31 U/l. The protein specific activity as measured in reconstituted yeast lyophilisate was 0.8 mU/mg protein. The recombinant enzyme exhibited similar Michaelis constants as previously determined for the native rat enzyme. By immunoblotting the enzyme was shown to be heterogeneous by size (44-48 kD) and *N*-glycosylated. We conclude that recombinant α2,6(*N*)sialyltransferase expressed in *S.cerevisiae* is retained in the endoplasmic reticulum as a fully active enzyme.

Complex carbohydrates exert many diverse biological functions (fo review see [1]). Their investigation requires availability of oligosaccharides. Up till now, chemical synthesis of complex carbohydrates is very labourious and inefficient [2]. The use of glycosyltransferases as tools for oligosaccharide synthesis is very attractive because of their regioselectivity and stereospecificity [2,3]. Due to their limited availability their application as catalysts for large-scale synthesis of oligosaccharides has not yet found wide application. In fact, no scaling-up of production of any glycosyltransferases has yet been published, except for α1,2-mannosyltransferase expressed in *E. coli* [4]. However, several reports on successful heterologous expression in yeasts and higher eukaryotic cells have already appeared [5].We have shown that expression of full length gal-T[1] in yeast is possible [6] and that scaling-up of expression of a soluble form led to a volume specific activity of 0.7 U/l [7-9]. Here we show that full length sialyl-T is also a good candidate for scaled-up expression in yeast. In this report we demonstrate the presence of sialyl-T in the yeast lyophilisate as an active enzyme

* Fax: (41) 1 364 0564.

Abbreviations: LacNAc, *N*-acetyllactosamine; CMP-NeuAc, Cytidine,5'-monophospho-*N*-acetylneuraminic acid; sialyl-T, α-2,6(*N*)sialyltransferase (E.C. 2.4.99.1); gal-T, β1,4galactosyltransferase (E.C. 2.4.1.22).

0006-291X/95 $5.00

having similar kinetic properties as the rat liver enzyme and bearing a glycosylation pattern compatible with its retention in an early compartment of the secretory pathway.

Experimental Methods

Materials

Yeast: The protease-deficient *Saccharomyces cerevisiae* strain BT150 (MATα, *his4, leu2, ura3, pra1, prb1, prc1, cps1*) (CIBA) was chosen for heterologous expression of sialyl-T. Plasmid: Construction of the expression plasmid pDPSIA was reported previously [10]. Briefly, this plasmid encodes full length human α2,6(*N*)sialyltransferase under the control of the constitutive PHO5 promoter/terminator. Antiserum: For immunoblotting, a rabbit polyclonal antiserum to a β-galactosidase- α2,6(*N*)sialyltransferase fusion protein expressed in *E. coli* was used as described previously [11].

Transformation and growth conditions

BT150 strain was transformed and grown as described previously [6]. For shaking flasks experiments *S. cerevisiae* BT150 was grown in the following medium: 6.7 g/l bacto-yeast nitrogen base without amino acids, 20 g/l glucose, 20 mg/l adenine sulfate, 20 mg/l tryptophane, 40 mg/l histidine, 20 mg/l arginine, 30 mg/l tyrosine, 60 mg/l leucine, 30 mg/l isoleucine, 30 mg/l lysine, 50 mg/l phenylalanine and 150 mg/l valine. The flasks were shaken at 30 °C and 150 rpm.

Scaling up of sialyl-T

Pilot scale fermentations were performed in a 300 l fermenter (Chemap, Volketswil, Switzerland) equipped with a pH-meter (Ingold, Germany), a fluorescent-detector (Ingold, Germany) and an oxygen pressure probe (amperometric detection, Ingold, Germany). The temperature was maintained at 30 °C and the stirrer speed was adjusted to 500 rpm. Oxygen partial pressure was kept constant at 60 % by aeration with air. The pH was adjusted to 5 with 4 M NaOH. Excess foaming was prevented by intermittent addition of antifoam. For pilot scale fermentation the glucose concentration was 100 g/l. Adenine sulfate and amino acids were used in a five fold higher concentration compared to shaking flask cultivation. Inoculation of the 150 l fermentation was 2.6% (v/v).

Harvesting and disintegration of the yeast

The cells were harvested in the stationary phase, cooled to 1 °C and separated from the medium with a self cleaning disk clarifier (Westfalia-Separator, Germany). The concentration of yeast cells was 42 % (v/v) after separation. In the next step the cells were washed once with icecold 1 % NaCl [11] and separated again. Cell disruption was performed in a 0.5 l glass bead mill (Disintegrator S, IMA-GmbH, Germany) containing 0.425 l 0.5 mm homogeneous glass beads in disruption buffer (50 mM Tris-HCl, pH 6.5). The cell concentrate (precooled to 4 °C) was pumped twice through the mill with a flow of 7.2 l/h. The mill speed was adjusted to 2500 rpm and the temperature was kept at 5 °C during disintegration through extensive cooling (temperature of the cooling liquid was -15 °C). Disintegrated cells were then lyophilized in presence of the disruption buffer.

α2,6(N)sialyltransferase activity assay

The yeast lyophilisate was reconstituted in H_2O to yield a protein concentration of 10 to 20 mg/ml affording approx. 40 mg of dry material. The assay contained in a final volume of 50 µl: 50 to 100 µg enzyme protein (at a concentration of 16.6 mg/ml), 50 mM Na cacodylate, pH 6.8, 0.5 % Triton X-100, 400 µM CMP-[^{14}C]-*N*-acetylneuraminic acid (final specific activity of 1.25 Ci/mol) obtained from Amersham (150-300 Ci/mol) mixed with unlabelled CMP-

Neu5Ac[1] (Oxford Glycosystems) and appropriate amounts of acceptor substrate. Acceptor substrates were either asialofetuin (Sigma), asialo-α_1 acid glycoprotein (gift from K.Schmid), or LacNAc[1] (Sigma). In the case of glycoprotein substrates, the reaction was stopped by addition of 0.5 ml of an ice-cold solution containing 5 % (w/v) phosphotungstic acid and 15 % (w/v) trichloroacetic acid. Precipitated protein was collected by suction filtration on Whatman GF/A glass fibre filters followed by 2 washes with ethanol. After filtration, the filter loaded with the precipitated [^{14}C] Neu5Ac-glycoprotein was dried and counted in 4 ml 0.8 % (w/v), 2-(*tert*-butylphenyl)-5-(4-bisphenylyl)-1,3,4,oxadialzole (CIBA) in toluene with a counting efficiency of 85 %. When LacNAc was used as an acceptor, the enzyme reaction was stopped by adding 1 ml icecold 5 mM Na_3PO_4 buffer, pH 6.8 and spun at 14'000 rpm for 5 min. The supernatant was passed over 1 ml AG1-X8 (Bio-Rad, phosphate form), washed with 1 ml of the same buffer. The effuent (2 ml) was collected in a plastic vial containing 10 ml Insta-Gel (Packard) and counted in a ß-scintillation counter. The standard incubation was 2 h at 37 °C. One unit corresponds to 1 µmol/min product formed under standard conditions.

Enrichment of enzyme for immunoblotting

Each of the following steps was carried out at 4 °C. Throughout the enrichment, protein was determined by the method of PIERCE using bovine serum albumin as a standard. Enzyme activity was measured as described above.

20 g of lyophilized yeast was dissolved in 400 ml H_20 containing 1% Triton X-100 and centrifuged at 30 000 *g* for 20 min. Supernatant was further purified by adjusting the pH to 6.5 and loading on a S-Sepharose column (600 ml) equilibrated with buffer A (50 mM Tris-HCl, pH 6.5, 0.5 % Triton X-100). The column was washed with buffer A /100 mM NaCl and the active fraction was eluted with buffer A/300 mM NaCl. The eluate (550 ml) was diluted 3 fold with 50 mM Tris-HCl, pH 6.5 and readsorbed on 100 ml S-Sepharose column (batch procedure), preequilibrated with buffer A/100 mM NaCl. Retained sialyl-T was eluted with a gradient of NaCl starting from 100 mM to 300 mM. Fractions containing activity were pooled and used for a second purification step after dialysis against buffer B (25 mM sodium cacodylate buffer, pH 6.5) containing 100 mM NaCl overnight. Dialyzed enzyme (20 ml) was loaded on a 1 ml CDP-hexanolamine-Sepharose 4B column (Genzyme) previously equilibrated with buffer B/100 mM NaCl. The column was washed with 10 volumes of buffer B/100 mM NaCl and eluted with 10 volumes of buffer B/500 mM NaCl [12], followed by concentration on an Amicon YM 30 membrane. Overall enrichment of specific activity was 286 fold yielding a preparation of 86 mU/mg.

Immunoblotting

Eluted fractions from S-Sepharose and CDP-hexanolamine-Sepharose were resolved on a 10 % SDS-polyacrylamide gel [13], either with or without endoglycosidase H treatment (Boehringer) according to the manufacturers instructions. Electrophoresis was followed by immunoblotting to nitrocellulose membrane as described previously [14]. Nitrocellulose membranes were incubated with anti-sialyltransferase antiserum diluted 200x followed by goat anti rabbit-alkaline phosphatase (1:1000, Biorad) and developed as described [14].

Results

To assess expression of recombinant sialyl-T in *S. cerevisiae* by activity and immunoblotting, expression was scaled-up in a 150 l bioreactor according to the protocol described above (figure 1). Under these conditions, volume specific activity reached a steady-state level after 40 h at approx. 0.31 U/l. This concentration corresponded to a mass specific activity of approx. 30 mU/g yeast wet weight or protein specific activity of 0.3 mU/mg protein. The yeast harvest

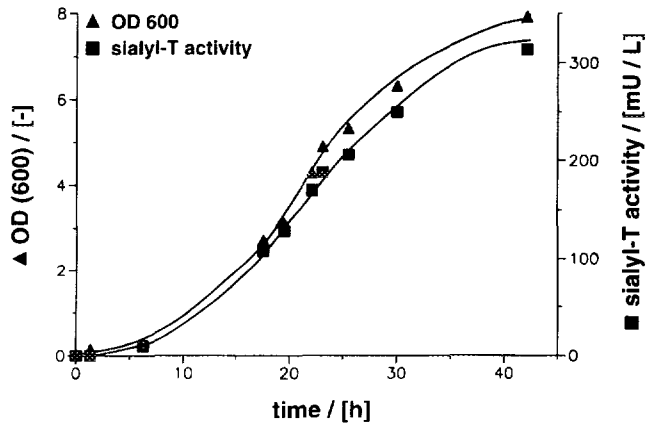

Fig.1. Heterologous expression of sialyl-T in a 150-liter fermentation. ▲, optical density at 600 nm (OD) [-]; ■, sialyl-T activity [mU/liter].

was lyophilized and used as a suspension in water without further purification for activity determinations. Sialyl-T activity assays were carried out according to the procedure described in the experimental section. We tested different acceptors in order to ascertain preservation of activity. Table 1 depicts the Michaelis constants determined for 3 acceptor and the donor substrates. These data correspond to those expected for a rat liver α2,6(*N*)sialyltransferase [15], which specifically sialylates terminal lactosaminoglycans. We conclude from these data that the recombinant sialyl-T as obtained after scaled-up expression and reconstituted from a crude yeast lyophilisate retains the properties previously published [15] and the recombinant human enzyme expressed at a small scale [10]. To further infer about the nature of the recombinant sialyl-T present in this yeast lyophilisate, the enzyme was enriched as described above for immunoblotting. Figure 2 shows that sialyl-T is expressed as a highly heterogeneous, glycosylated enzyme migrating as a broad band between 44 to 48 kD. Treatment with endoglycosidase H converted the heterogeneous band to enzyme forms smaller by approx. 4 kD without apparent changes in their heterogeneity or immunoreactivity. We therefore conclude that all sialyl-T expressed at steady-state and enriched as described above, occurred in a glycosylated form substituted with oligomannosidic *N*-glycans. This type of posttranslational modification indicates retention of active sialyl-T in an early compartment of the secretory pathway.

Discussion

This report deals with the second example of scaled-up expression of a mammalian glycosyltransferase in the yeast expression system. Previously we have described expression of gal-T in the same protease-deficient yeast strain of *S. cerevisiae* [6] and its scaling-up as a

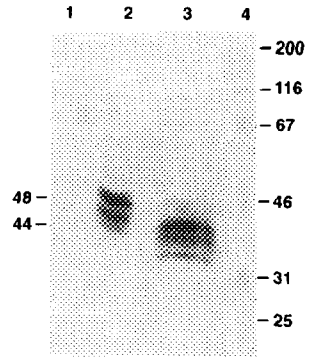

Fig. 2. Immunoblot of a partially purified recombinant membrane sialyl-T from yeast. The lanes were loaded as follows:
(1) Eluate from S-Sepharose column; (2) Eluate from CDP-hexanolamine-Sepharose; (3) Eluate from CDP-hexanolamine-Sepharose after treatment with endoglycosidase H; (4) prestained molecular weight standards (Biorad).

soluble enzyme [7]. Interestingly, in this latter case, a volume-specific activity up to 0.7 U/l was reached, thus an approx. 2.5 times higher concentration as in the present case. Since host cells and plasmids were the same except for the inserted cDNA, the difference may relate to the fact that in the case of soluble gal-T, the enzyme was more efficiently transported through the secretory pathway as indicated by hyperglycosylation of its single *N*-glycosylation site (Malissard et al. manuscript in preparation). No evidence of hyperglycosylation was obtained in the case of expression of full length (membrane-bound) sialyl-T, neither by metabolic labeling [9] nor by immunoblotting (Figure 2). The fact that *N*-deglycosylation of sialyl-T did not reveal additional immunoreactive material further supports our assumption that sialyl-T was not hyperglycosylated and, therefore, has not been modified by the elongating mannosyltransferase located in the Golgi apparatus. Based on these data and on previous results obtained by pulse/chase analysis of the metabolically labeled enzyme [10], we conclude that sialyl-T was retained as an active enzyme in the endoplasmic reticulum or in a very early Golgi compartment. Sensitivity to endoglycosidase H ascertains our conclusion of full translocation competence of the sialyl-T protein since all of it appeared to be *N*-glycosylated. On the basis of the results presented here, the nature of the size heterogeneity of the enzyme remains unclear. Possible explanations may include variable O-mannosylation or limited proteolytic degradation or both.

The slight differences in kinetic data to those published previously [10] may relate to the extensive heterogeneity as revealed by immunoblotting. They indicate proper folding of the enzyme and little,if any impairment of catalytic activity by *N*-glycosylation. This may, however, not be true in the case of a soluble form of sialyl-T. In analogy to expression of gal-T [6,8], a soluble form of sialyl-T was prepared by truncation at the luminal side of the transmembrane

domain, linked in frame with the invertase signal sequence and expressed in yeast (our unpublished data). Expression of soluble sialyl-T as an active enzyme was possible but only very low activity was detectable by virtue of an assay based on acceptor substrates substituted with a hydrophobic aglycone[16]. Thus, activity of soluble sialyl-T might be obscured by Golgi assosiated *N*-hyperglycosylation, since one of the two *N*-glycosylation sites is located close to the catalytic portion [17].

Importantly, scaled-up production of sialyl-T contributes to a reduction of animal use for commercialization of this enzyme as a fine biochemical. Currently sold sialyl-T is obtained from rat liver according to a procedure described by Weinstein [18]. We estimate that a 150 l fermentation spares approx. 150 rats.

In summary, we show that bulk expression of functionally active, full length α2,6(*N*)sialyltransferase is possible in *S. cerevisiae* and that this enzyme will prove useful for the *in vitro* synthesis of sialyl-oligosaccharides.

Acknowledgments

This work was supported by a grant 2305.1 of the KWF to EGB and a grant of the Medical Faculty of the University of Zürich.

References

[1] Varki, A. (1993) Glycobiology 3, 97-130.
[2] Ichikawa, Y., Look, G. C., Wong, C. H. (1992) Anal. Biochem. 202, 215-238.
[3] Meldal, M. (1994) Curr. Opin. Structur. Biol. 4, 710-718.
[4] Herrmann, G.F., Wang, P., Shen, G.-J., Garcia-Junceda, E., Khan, S.H., Matta, K.L., Wong, C.-H. (1994) J. Org. Chem. 59, 6356-6362.
[5] Kleene, R., Berger, E.G. (1993) Biochim. Biophys. Acta. 1154, 283-325.
[6] Krezdorn, C.H., Watzele, G., Kleene, R.B., Ivanov, S.X., Berger, E.G. (1993) Eur. J. Biochem. 212, 113-120.
[7] Herrmann, G.F., Krezdorn, C., Malissard, M., Kleene, R., Paschold, H., Weuster-Botz, D., Kragl, U. Berger, E.G., Wandrey, C. (1995) Protein Express. Purif. 6, 72-80.
[8] Kleene, R., Krezdorn, C.H., Watzele, G., Meyhack, B., Herrmann, G.F., Wandrey, C., Berger, E. G. (1994) Biochem. Biophys. Res. Commun. 201, 160-167.
[9] Herrmann, G.F., Elling, L., Krezdorn, C.H., Kleene, R., Berger, E.G., Wandrey, C. (1995) Bioorg. Medical Chem. Letter, in press.
[10] Krezdorn, C.H., Kleene, R.B., Watzele, M., Ivanov, S.X., Hokke, C. H., Kamerling, J.P., Berger, E.G. (1994) Eur. J. Biochem. 220,809-817.
[11] Berger, E.G., Grimm, K., Bächi, T., Bosshart, H., Kleene, R., Watzele, M. (1993) J. Cell. Biochem. 52, 275-288.
[12] Paulson, J.C., Beranek, W.E., Hill, R.L. (1977) J. Biol. Chem. 252, 2356-2362.
[13] Laemmli, U.K. (1970) Nature 227,680-685.
[14] Watzele, G., Bachofner, R., Berger, E.G. (1991) Eur. J. Cell Biol. 56, 451-458.
[15] Wlasichuk, K.B., Kashem, M.A., Nikrad, P.V., Bird, P., Jiang, C., Venot, A.P. (1993) J. Biol. Chem. 268,13971-13977.

[16] Palcic, M.M., Heerze, L.D., Pierce, M., Hindsgaul, O.(1988) Glycoconjugate J. 5, 49-63.
[17] Grundmann, U., Nerlich, C., Rein, T., Zettlmeissl, G. (1990) Nucl. Acid Res. 18, 667-668.
[18] Weinstein J., de Souza-e-Silva, U., Paulson, J.C. (1982) J. Biol. Chem. 257, 13835-13844.
[19] Horowitz, M.I., Pigman, W. (1977) in: The glycoconjugates Vol. I Mammalian glycoproteins and glycolipids (Schwick, H.G., Heide, K., Haupt, H. eds) Blood glycoproteins pp.262-350, Academic Press Inc., London, New York.