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Chemo-enzymatic synthesis of a selectin ligand using recombinant yeast cells

André Lubineau [a], Christine Le Narvor [a], Claudine Augé [a,*], Paul François Gallet [b], Jean Michel Petit [b], Raymond Julien [b]

[a] *Laboratoire de Chimie Organique Multifonctionnelle, URA CNRS 462, Université Paris-Sud, 9145 Orsay Cedex, France*
[b] *Institut de Biotechnologie, Unité Associée de Génétique Moléculaire Animale, Université de Limoges, 87060 Limoges Cedex, France*

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Abstract

Functional soluble human α(1-3/4) fucosyltransferase was successfully expressed in *Pichia pastoris* cells. The recombinant protein was located in the periplasmic space; thus, incubation of the whole yeast cells with disulfated tetrasaccharide **1** (SO_3^-3)Galβ1-4(SO_3^--6)GlcNAcβ1-3Galβ1-4GlcβOMBn and GDP-fucose directly provided pentasaccharide **2** (SO_3^-3)Galβ1-4(Fucα1-3)(SO_3^--6)GlcNAcβ1-3Galβ1-4GlcβOMBn in 70% yield.

Keywords: Chemo-enzymatic synthesis; *Pichia pastoris*; α(1-3/4) Fucosyltransferase

1. Introduction

Due to their regio and stereoselectivity, enzymes have proven to be a powerful tool as catalysts in carbohydrate chemistry avoiding the need of protection and deprotection steps [1,2]. However, in oligosaccharide synthesis the major drawback is the lack of availability of natural glycosyltransferases that are isolated from natural sources only in limited amounts. For this reason, a great amount of interest has been directed toward the cloning of the glycosyltransferase genes into convenient expression systems. In particular, several fucosyltransferases with different specificities have been cloned [3]; fucosyltransferases catalyze transfer of fucose, one of the last sugar residue introduced in vivo at the non-reducing end of oligosaccharide chains of glycoproteins and glycolipids, from GDP-fucose, the nucleoside-diphospho-sugar donor, according to this scheme:

GDP-Fuc + oligosaccharide-acceptor

→ Fucosyl-oligosaccharide-acceptor + GDP

They are involved in the biosynthesis of most important bioactive oligosaccharides: thus sulfated (or sialylated) and fucosylated oligosaccharides have been recently shown to be ligands for E, P and L-selectins, a family of adhesion molecules that mediate the interaction of circulating leukocytes with endothelial cells in recruitment to sites of inflammation [4,5].

* Corresponding author. Fax: +33-0-1-69-15-47-15; E-mail: clauauge@icmo.u-psud.fr

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These recent advances prompted us to synthesize for immunological studies the disulfated pentasaccharide $(SO_3^-3)Gal\beta 1\text{-}4(Fuc\alpha 1\text{-}3)(SO_3^-\text{-}6)GlcNAc\beta 1\text{-}3Gal\beta 1\text{-}4Glc$ (so-called $3^{IV}\ 6^{III}$ Lewis^x pentasaccharide), according to a chemo-enzymatic approach, relying on the use of natural galactosyl and fucosyl transferases [6].

Here, we report the expression of functional soluble $\alpha(1\text{-}3/4)$ fucosyltransferase (*FUT3*) in *Pichia pastoris* cells and describe the use of the transformed whole cells, instead of the natural human milk enzyme, in the fucosylation of the disulfated tetrasaccharide **1** $(SO_3^-3)Gal\beta 1\text{-}4(SO_3^-\text{-}6)GlcNAc\beta 1\text{-}3Gal\beta 1\text{-}4Glc\beta O$MBn. [1]

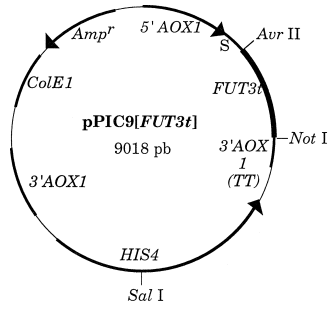

Fig. 1. Human $\alpha(1,3/4)$ fucosyltransferase expression plasmid pPIC9[*FUT3t*]. A 1002 pb DNA fragment encoding the truncated human $\alpha(1,3/4)$ fucosyltransferase protein (*FUT3t*) was cloned in *Avr*II/*Not*I sites between the α-factor signal sequence (S) and the 3′*AOX1* transcription termination fragment (3′*AOX1* (TT)). In this way, *FUT3t* was under the control of the *AOX1* promoter (5′*AOX1*). The vector also contains the histidinol dehydrogenase gene (*HIS4*), 3′ *AOX1* sequences (3′*AOX1*), the ampicillin resistance gene and other sequences necessary for propagation in *E. coli*.

2. Experimental

2.1. Materials and methods

Plasmid pPIC9, *P. pastoris* strain GS115 (*his4*) were obtained from InVitrogen (San Diego, CA). Enzymes for DNA manipulations were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany) or New England Biolabs (Beverly, MA). Yeast nitrogen base was purchased from Difco Laboratories (Detroit, MI). All other general methods were as described [5].

2.2. Construction of the pPIC9[FUT3t] vector

The truncated *FUT3* gene was excised from the pFUT3 vector [7] as a *Avr*II/*Not*I fragment and ligated into the same sites in the MCS of pPIC9 giving the pPIC9[*FUT3t*] plasmid (Fig. 1; Table 1). This was linearized with *Sal*I and inserted into the yeast host strain GS115 (*his4*). Homologous recombination occurs between *HIS4* sequences of the vector and those of the host genome. The integration at the correct locus was screened by growth on histidine-deficient medium (1.34% (w/w) YNB, 4×10^{-5}% (w/w) biotin and 0.5% (v/v) methanol). Clones which grew rapidly on this medium were selected and tested for production.

2.3. Fucosyltransferase production

One liter of fermentation medium corresponded to 0.67% (w/w) YNB and 4×10^{-5}% (w/w) biotin. The pH was maintained at 6.0 with 28% (v/v) ammonium hydroxide. Methanol was continuously added at a flow of 5 g/l h. Agitation speed was 600 rpm, air flow was maintained at 2.5 l/min. Temperature was maintained strictly at 30°C.

To test fucosyltransferase activity, cells were broken using glass beads [8] in a medium containing sodium cacodylate 20 mM, 1% triton X100 (v/v), pH 6. The homogenate was centrifuged at $10\,000\times g$, 5 min and 4°C, and the fucosyltransferase assay was done with 50 μg of the supernatant protein according to Ref. [9], using GDP − [^{14}C]fucose (Amersham, 300 mCi/mmol) and 6 μl of 1 mg/ml solution of $Gal\beta 1\text{-}3GlcNAc\beta O(CH_2)_7\text{-}CH_3$ **3**.

[1] The abbreviations used are Fuc for fucose, Gal for galactose, Glc for glucose, GlcNAc for *N*-acetyl-glucosamine; all sugars belong to D serie, except fucose that belongs to L serie.

Table 1
Acceptor specificity of *FUT3t* expressed in *P. pastoris* and tested as whole cells

Substrates	Concentration (mM)	Relative activity (%)
Galβ1-3GlcNAcβ*O*octyl	0.6	100
Galβ1-4GlcNAcβ*O*benzyl	5	8
	10	25
	20	34
Galα1-3Galβ1-3GlcNAcβ*O*octyl	0.6	190

2.4. Assay for FUT3 with whole recombinant cells

The yeast cells were harvested, directly lyophilized, stored as a lyophilizate at 4°C, and used as a suspension in buffer for enzymatic assay. The assay was performed with **3** (type 1 disaccharide) as the acceptor substrate (0.6 mM) in a final volume of 50 μl containing 3 mM ATP, 20 mM $MnCl_2$, 0.14 mM GDP – [^{14}C – fucose] (38 000 cpm), 50 mM cacodylate buffer pH 7.4, 0.5 mg of lyophilized cells, and incubated at 37°C with gentle stirring. The reaction was stopped by adding 1 ml ice-cold water, applied to 1 ml Dowex 1-X8 pipet column and washed once with 1 ml water. The flow-through and the column washes were collected in 10 ml of scintillation cocktail. The relative activity towards Galβ1-4GlcNAcβ*O*Bn (type 2 disaccharide) was measured in the same conditions but for 5, 10 and 20 mM substrate concentrations.

*2.5. Large-scale incubation and characterization of the fucosylation product **4***

Disaccharide **3** (5 mg, 10 μmol) and GDP-Fuc [10] (7 mg, 11 μmol) were incubated at 37°C with gentle shaking in 50 mM sodium cacodylate buffer (0.5 ml) containing 20 mM $MnCl_2$, 5 mM ATP and 100 mg of lyophilized cells (5 mU). After one day, more GDP-Fuc (5 mg, 8 μmol) was added and the reaction was pursued for 2 days. Then EtOH (1 ml) was added to the reaction, the mixture was centrifugated, the precipitate washed with water and the supernatant and washings were collected and applied to a column of BioBeads-SM2 (Bio-Rad). After washing with water, disaccharide **3** and trisaccharide **4** were eluted together with 1:9 2-propanol-water; 50% conversion, ^{1}H-NMR (CD_3OD) for **4**: δ 4.93 (d, 1H, *J* 3.8 Hz, H-1^{III}), 4.41 (d, 1H, *J* 8 Hz, H-1^{II}), 4.27 (d, 0.5 H, *J* 7.5 Hz, H-1^{I}), 1.85 (s, 3H, NHAc), 1.40 (m, 2H, CH_2), 1.18 (m, 10H, 5 CH_2), 1.06 (d, 1H *J6* Hz, CH_3^{III}), 0.83 (t, 3H, CH_3).

*2.6. 4-Methoxybenzyl O-(3-sulfo-β-D-galactopyranosyl-(1 → 3)-O-[(α-L fuco-pyranosyl)(1 → 4)] -O- (-2- acetamido -2- deoxy -6-O-sulfo-β-D-glucopyranosyl)-(1 → 3)-O-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside disodium salt (**2**)*

The disulfated tetrasaccharide **1** (5 mg, 4.9 μmol) and GDP-Fuc (3.5 mg, 5.5 μmol) were incubated at 37°C in 50 mM sodium cacodylate buffer (0.5 ml) containing 20 mM $MnCl_2$, 5 mM ATP and 600 mg of lyophilized cells (30 mU) with gentle shaking. After 1 and 2 d, more GDP-Fuc (7 mg, 11 μmol, altogether) were added, and the incubation was allowed to continue for 4 days. The reaction was worked up as previously described for fucosylation of **3**. Supernatant from EtOH precipitate and washings were applied to a column of DEAE-Sephadex A25 (HCO_3^- form). Elution with a gradient of 0 to 2 M triethylammonium hydrogen carbonate buffer (pH 8.0) afforded a mixture of tetrasaccharide **1** and pentasaccharide **2** as their bis(triethylammonium) salt. Further flash chromatography in 6:6:1 2-propanol-EtOAc-H_2O yielded pure **2**, which was converted to the disodium salt by passage down a column of Bio-Rad AG

50W-X8 resin (Na^+ form). The freeze-dried eluate afforded **2** (4.0 mg, 70%); $[\alpha]_D^{26}$-21°(*c* 0.5, H_2O); ^1H NMR (D_2O, 250 MHz): δ 7.35 (d, 2H *J* 8 Hz, Ph), 6.98 (d, 2H, Ph), 5.08 (d, 1H, *J* 4 Hz, H-1^V), 4.87 (q 1H, *J* 6 Hz, H-5^V), 4.71 (d, 1H, *J* 8 Hz, H-1^{III}) 4.64 (d, 1H, *J* 11 Hz, Ph-CH), 4.57 (d, 1H, *J* 8 Hz, H-1^{IV}), 4.47 (d, 1H, *J* 8 Hz, H-1^I), 4.37 (d, 1H, *J* 8 Hz, H-1^{II}), 4.32 (m, 2H, H-6^{III}, H-$6'^{III}$), 4.24 (dd, 1H, *J* 3, *J* 10 Hz, H-3^{IV}), 4.22 (d, 1H, H-4^{IV}), 4.13 (d, 1 H, *J* 3 Hz, H-4^{II}), 3.82 (s, 3H, OCH_3), 3.26 (dd, 1H, H-2^I), 2.0 (s, 3H, OAc) and 1.15 (d, 3H, CH_3); LRMS (negative mode): *m/z* 565.6 $[M\text{-}2\ Na]^{2-}$.

3. Results and discussion

The methylotrophic yeast *P. pastoris* can be grown to high cell density and support high expression levels of heterologous protein [11]. Heterologous protein expression is regulated by the very strong alcohol oxidase (*AOX1*) promoter. The cloned gene could be inserted directly into the yeast genome, thereby avoiding potential plasmid instability. In addition, *P. pastoris* secretes heterologous proteins through a secretory pathway utilizing an appropriate secretion signal [11]. This permits post-translational modifications such as proteolytic maturation, glycosylation, native folding, and disulfide bound formation.

For expression of the recombinant truncated human $\alpha(1,3/4)$ fucosyltransferase-secreted form, DNA fragment encoding the enzyme was truncated of its 132 first nucleotides (44 first amino acids) to rule out the transmembrane anchoring domain of the native enzyme. It was inserted downstream of the alcohol oxidase (*AOX1*) promoter, resulting in plasmid pPIC9[*FUT3t*]. As a consequence of the use of the yeast signal sequence (*a*-factor signal sequence) and signal peptidases cleavage sites to release the recombinant enzyme, a 37.7-kDa protein with four extra amino acid residues at the NH_2 terminus compared to the natural sequence (Tyr-Val-Glu-Phe-Pro-Arg-Ala… instead of Pro-Arg-Ala…) was obtained (Fig. 1).

Production of truncated enzyme was done for 48 h. During this induction time, no fucosyltransferase activity was detected in culture broth, but was only found in cell homogenate instead (Fig. 2). Results displayed in Fig. 2 show that a very low specific activity could be detected until the culture time reached 30 h. Then activity increased with time until 48 h. Moreover, activity measured in homogenates could be entirely found again by using undisrupted cells. Thus, it was evident that the enzyme remained located in the periplasmic space. This assumption was confirmed by cell fractionation and releasing of fucosyltransferase activity following cell wall digestion by lyticase (data not shown).

This observation prompted us to use the whole yeast cells instead of the cell homogenate for enzymatic synthesis. Thus, in the radioactive assay, the *FUT3* activity tested with the disaccharide Galβ1-3GlcNAcβ*O*octyl (so called type 1 disaccharide) as the substrate was 50 mU/g dried cells. The activity was also tested with the isomeric disaccharide Galβ1-4GlcNAcβ*O*benzyl (so-called type 2 disaccha-

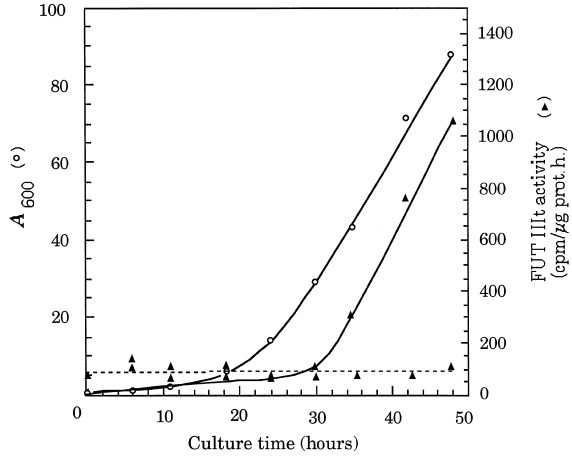

Fig. 2. Expression of truncated human $\alpha(1,3/4)$ fucosyltransferase in *P. pastoris* strain (GS115). The production of enzyme was evidenced by measuring fucosyltransferase activity (▲) of cell homogenates (full line) and culture broth (broken line); (– ○ –): cell growth.

ride). It should be mentioned that because of insolubility in water the octyl glycoside of type 2 disaccharide first considered, had to be replaced by the benzyl glycoside. As shown in the table, the type 2 disaccharide at 5 mM concentration was only 8% as effective an acceptor as the type 1 disaccharide. With higher substrate concentrations, the relative activity can be increased up to 34%. The same preference for the type 1 disaccharide has been observed for the natural human milk $\alpha(1\text{-}3/4)$ fucosyltransferase [12]. Moreover, the type 1 disaccharide substituted by a galactose residue at the non-reducing end exhibited a much better activity that the parent disaccharide (190% relative activity).

In order to fully characterize the fucosylation product, incubation of disaccharide **3** with *Pichia* cells was achieved on a 5-mg scale using non-radiolabelled GDP-fucose, prepared according to the published procedure [10]. The reaction was stopped after two days, and the mixture of substrate and product could be readily purified by reverse phase chromatography. From ^{1}H NMR spectrum, a **3**:**4** ratio of 1:1 was estimated. ^{1}H NMR data for trisaccharide **4** were in good accordance with the literature [13], and thus confirmed that the fucose had been transferred to the O-4 position of the *N*-acetylglucosamine residue (Fig. 3).

Encouraged by this result, we undertook fucosylation of disulfated tetrasaccharide **1**. To this end, substrate **1** was incubated at 20 mM

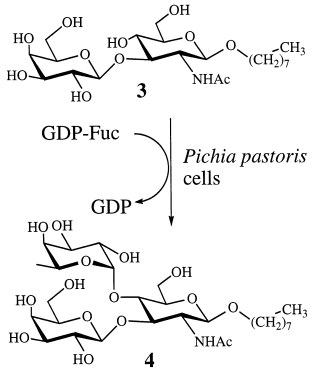

Fig. 3. Fucosylation of disaccharide **3** with transformed whole *Pichia* cells leading to trisaccharide **4**.

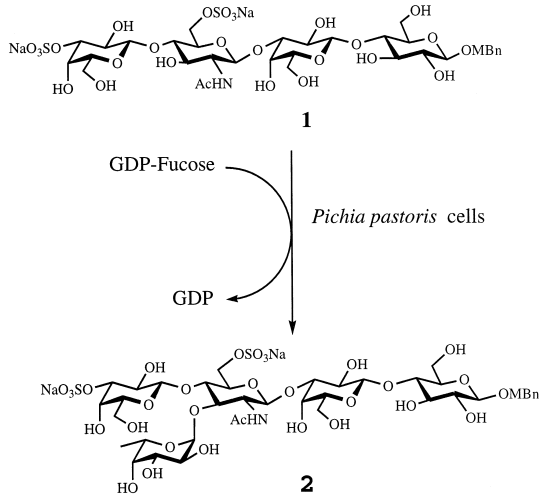

Fig. 4. Fucosylation of disulfated tetrasaccharide **1** using transformed whole *Pichia* cells.

concentration with GDP-fucose and *Pichia* cells, in the same conditions than **3** (Fig. 4). The reaction was stopped after 4 days, when HPTLC analysis showed a major spot corresponding to the reference pentasaccharide and no more evolution. The disulfated pentasaccharide **2** was first purified by anion exchange chromatography, elution with volatile triethylammonium hydrogen carbonate, affording **2** contaminated by remaining starting tetrasaccharide **1**. Subsequent silica gel chromatography led to pure **2** (70%) characterized by NMR analysis. ^{1}H NMR spectrum of **2** matched completely the one previously reported [6].

4. Conclusion

We demonstrated here the efficient expression of human $\alpha(1,3/4)$ fucosyltransferase in the methylotrophic yeast *P. pastoris*. The enzyme was truncated by deletion of its cytoplasmic and transmembrane domains. The recombinant protein was located in the periplasmic space; however, it is worth mentioning that for an induction time longer than 48 h, fucosyltransferase activity could be detected in the culture medium. The fact that the enzyme re-

mained first located in the periplasmic space was exploited by using directly the transformed whole cells for synthetic purpose: such procedure circumvented tedious enzyme purification. *Saccharomyces cerevisiae* cells expressing recombinant galactosyltransferase have been previously used, in a similar way, as catalysts in the synthesis of *N*-acetyllactosamine [14].

Acknowledgements

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