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Outstanding stability of immobilized recombinant α(1→3/4)-fucosyltransferases exploited in the synthesis of Lewis a and Lewis x trisaccharides

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Recombinant human α(1→3/4)-fucosyltransferases (FucT-III) expressed in CHO cells or baculovirus-infected insect cells, immobilized on Ni^{2+}-agarose through a 6His tag, exhibit a marked stability, which was exploited in the synthesis of Lewis a and Lewis x trisaccharides.

Glycosyltransferases have become widely used over the past decade as efficient tools for glycosylation because they catalyze sugar unit transfer with complete regio- and stereoselectivity. Early work in this area suffered from the low availability of these membrane-bound enzymes isolated from natural sources, but nowadays, thanks to genetic engineering, the expression of secreted forms of recombinant glycosyltransferases offers the opportunity of producing active enzymes in large quantities. Fucosyltransferases comprise a family of enzymes that catalyze the transfer of a fucose residue from GDP-fucose, the sugar-nucleotide donor, to a disaccharide acceptor. Among this family, α(1→3/4)-fucosyltransferase (FucT-III), is responsible for both the synthesis of the Lewis a trisaccharide Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-GlcNAc*p* from β-D-Gal*p*-(1→3)-β-D-GlcNAc*p* (type 1 disaccharide), and to a smaller extent the synthesis of the Lewis x trisaccharide Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-GlcNAc*p* from β-D-Gal*p*-(1→4)-β-D-GlcNAc*p* (type 2 disaccharide). FucT-III, consist of a short NH_2-terminal cytoplasmic tail, a transmembrane domain and a stem region followed by a large globular COOH-terminal catalytic domain. Previous works have reported cloning of the human FucT-III, expression of a soluble form in COS cells and in BHK-21 cell lines.[1] Besides, another construct of recombinant FucT-III turned out to be very efficient to produce sialyl Lewis a libraries.[2]

Within a French network devoted to the production and studies of recombinant glycosyltransferases, the cDNA coding for the human FucT-III gene[1] deleted of the part corresponding to the transmembrane domain was again expressed in three different expression systems: *Pichia pastoris* yeast,[3] Chinese Hamster Ovary (CHO) cells and baculovirus-infected insect cells. In CHO and baculovirus-infected insect cells, a 6His tag was added to the sequence, at the *C*-terminal end in the first case, at the *N*-terminal end in the latter case.[4]

We wish to report here an efficient procedure for concentration, immobilization and stabilization of FucT-III, relying on the His tag of the recombinant enzymes.

In each expression system, CHO and baculovirus-infected insect cells, a functional soluble form of FucT-III was produced. The specificities of both recombinant enzymes were compared (see Table 1). The highest activity was noticed when type 1 disaccharide was substituted by a galactose residue. Activity with the type 2 acceptor increased when concentration changed from 10 to 20 mM, which clearly showed evidence of the low affinity for β-D-Gal*p*-(1→4)-β-D-GlcNAc*p* and β-D-Gal*p*-(1→4)-β-D-Glc*p* disaccharides. No significant difference in substrate specificity has been observed between both FucT-III. Furthermore, a novel fluorescent assay was developed as an alternative to radioactive assays routinely used for glycosyltransferases. For this purpose, new disaccharide derivatives **3**

Table 1 α1,4 and α1,3 activities of recombinant FucT-III expressed in CHO or baculovirus-infected insect cells on various acceptors

	CHO Vrel[a]	Baculovirus Vrel[a]
β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-$O(CH_2)_7CH_3$[c]	100	100
α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-GlcNAc*p*-$O(CH_2)_7CH_3$[c]	125	112
α-D-NeuAc*p*-(2→3)-β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-OBn[c]	42	53
β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-OBn[b]	9.5	4.5
β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*[c]	25	15
β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*[d]	38	38
β-D-Gal*p*-(1→4)-β-D-Glc*p*[c]	37	26
β-D-Gal*p*-(1→4)-β-D-Glc*p*[d]	50	46

[a] Relative velocities with 0.14 mM GDP-[^{14}C]Fuc. [b] Tested at 6 mM because of low solubility in water. [c] Tested at 10 mM. [d] Tested at 20 mM.

Table 2 Kinetic constants of recombinant FucT-III expressed in CHO and baculovirus-infected insect cells, towards dansyl disaccharides **3** and **4**. Enzyme assay conditions with fluorescent acceptors: 50 mM sodium cacodylate buffer pH 7.4, 5 mM ATP, 20 mM $MnCl_2$, 0.2 mM GDP-Fuc, 0.05 to 1 mM disaccharide **3** or **4**. The mixture was incubated at 37 °C for 15 to 60 min with 10-fold concentrated culture supernatant. Samples were analyzed by HPLC on a Waters 600 equipped with a reversed phase column (Nucleosil). Detection was done with a Luminescence Spectrometer LS50B from Perkin–Elmer. Fluorescence of substrate and product was read at 385 nm excitation/540 nm emission.

	Baculovirus	CHO
Vmax for **3**, mU mL^{-1}	22.5	15.5
KM for **3**, mM	0.9	0.4
Vmax for **4**, mU mL^{-1}	2[b]	Nd[a]
KM for **4**, mM	9.9[b]	Nd[a]

[a] Nd: not determined. [b] Because of low solubility of disaccharide **4** in water, 5% DMSO was added.

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Table 3 Immobilization of recombinant FucT-IIIs on Ni^{2+}-NTA-agarose. Culture supernatant (23 mL) was immobilized on 1 mL of gel

	Initial soluble enzyme activity mU mL^{-1a}	Gel-bound enzyme activity mU mL^{-1a}	Immob. yield %[b]
CHO	1.3	28	92
Baculovirus	2.1	41	87

[a] U represents the enzyme unit number defined as the quantity of enzyme that catalyzes transformation of 1 μmole of substrate per min. [b] Immobilization yield is expressed as the ratio of immobilized activity to that initially present in the solution.

and **4** bearing, an aminohexyl chain substituted by a dansyl group at the anomeric center were prepared. Kinetic parameters for these substrates are reported for both enzymes in Table 2.

Recombinant FucT-III were tagged with a polyhistidine tail: indeed a stretch of six His is now commonly appended to the primary sequence of recombinant proteins, in order to facilitate their purification by Ni^{2+} affinity chromatography, according to strong interactions between Ni^{2+} immobilized on agarose through ligands such as nitriloacetic (NTA) and the polyhistidine tag.[5] Thus the 6His-tagged FucT-IIIs were immobilized on Ni^{2+}-NTA-agarose at 4 °C according to a batch procedure. Prior to the binding step, the culture media must be dialyzed, in order to adjust the pH and remove any interfering components. Irrespective of the position of the His tag, the *N*-terminal and *C*-terminal tagged FucT-IIIs were immobilized in quantitative yield (see Table 3). However it is worth noting that attempts to elute enzymes from the gel were troublesome and unsuccessful; imidazole turned out to be a strong inhibitor of FucT-III, and less than 5% of enzyme activity was recovered in the dialyzed eluate. On the contrary, the immobilisation of FucT-III on Ni^{2+}-agarose presented two major advantages. First it turned out to be a very efficient procedure for concentrating enzyme activity (by a 15–20 factor), much better than ultrafiltration bringing about some protein denaturation. Secondly immobilized FucT-III exhibited an outstanding stability under enzymatic incubation conditions at 37 °C with an half-life of three weeks (Fig. 1). On the other hand, CHO culture supernatant incubated in the same conditions proved to be very unstable (Fig. 1).

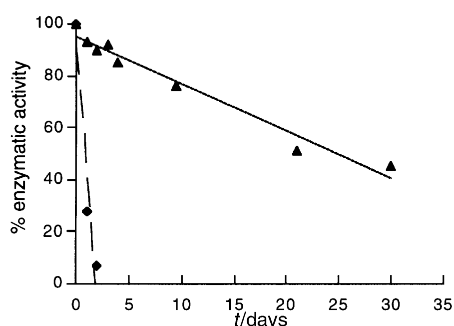
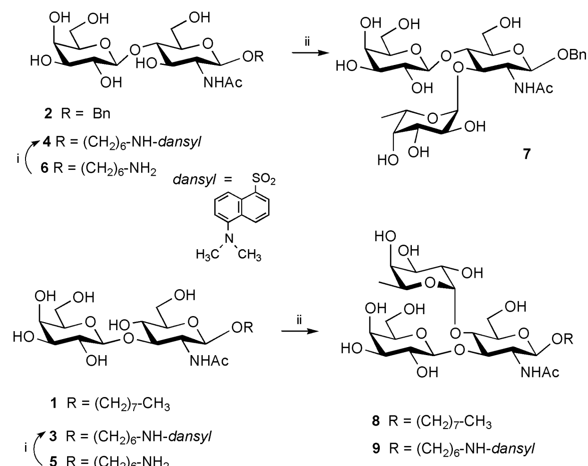


Fig. 1 Percentage of remaining enzymatic activity as a function of incubation time, expressed in days, for FucT-III immobilized on Ni^{2+}-NTA-agarose (▲) and crude CHO culture supernatant (◆) both incubated at 37 °C in 0.1 M sodium cacodylate buffer pH 7.4.

Disaccharide **2** was incubated with GDP-fucose and Mn^{2+} in the presence of the recombinant CHO FucT-III adsorbed on Ni^{2+}-agarose to afford the Lewis x trisaccharide **7** (Scheme 1). Fucosylation of this disaccharide required a long incubation time (10 d) because of its low affinity, but could finally be achieved in 80% yield. At the end of the incubation almost 50% of enzyme activity still remained. Then the same immobilized enzyme could be used two more times for the synthesis of Lewis a trisaccharides **8** and **9** in quantitative yields from the best disaccharide substrates **1** and **3** (Scheme 1).‡

To our knowledge there was one previous report on the use of a recombinant mannosyltransferase immobilized on Ni^{2+}-agarose for synthesis on a microscale, but the increased stability of the immobilized biocatalyst was not highlighted.[6]



Scheme 1 *Reagents*: i, dansyl chloride, Na_2CO_3, acetone; ii, FucT-III adsorbed on Ni^{2+}–agarose, GDP-Fuc, 5 mM $MnCl_2$.

Work is now in progress to extend immobilisation on Ni^{2+} to other His-tagged recombinant glycosyltransferases and to apply such immobilized enzymes to the supported enzymatic synthesis of oligosaccharides.

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Notes and references

‡ Benzyl-β-D-*N*-acetyllactosamine **2** (0.015 g, 0.032 mmol) was incubated at 37 °C with FucT-III immobilized on Ni^{2+}-NTA-agarose (0.070 U) and GDP-Fuc (0.047 in sodium cacodylate buffer pH 7.4 (3 mL) containing 0.01% NaN_3, $MnCl_2$ (0.015 mmol) and intestine phosphatase (4 U). At the end of incubation, the gel was filtered off and the filtrate was applied onto Sep-Pak C_{18} cartridges; the products were eluted from Sep-Pak with MeOH. The gel could be recycled twice, first with disaccharide **1** (0.032 mmol), secondly with disaccharide **3** (0.016 mmol).

Trisaccharide **7**: 80% yield; δ_H (CD_3OH) 1.10 (d, 3 H, $J_{5,6}$ 6.5 Hz, CH_3), 1.85 (s, 3 H, NAc), 4.41 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.52 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.62 (d, 1 H, *J* 12 Hz, C-H), 5.00 (d, 1H, $J_{1'',2''}$ 4 Hz, H-1″), 7.35 (5 H, Ph). LRMS: calc. for $C_{27}H_{41}O_{15}N$: *m/z* 619.6; found 642.3 (*M* + Na^+).

Trisaccharide **8:** quant. yield; δ_H (CD_3OH) 0.8 (t, 3 H, CH_3), 1.11 (d, 3 H, $J_{5,6}$ 6.5 Hz, CH_3), 1.22 (m, 12 H, 6 CH_2), 1.48 (m, 2 H, CH_2), 1.98 (d, 3 H, NAc), 4.43 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.45 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1′), 4.96 (d, 1H, $J_{1'',2''}$ 3.Hz, H-1″). LRMS: calc. for $C_{28}H_{51}O_{15}N$: *m/z* 641.7; found 664.3 (*M* + Na^+).

Trisaccharide **9**: quant. yield; δ_H (CD_3OH) 1.18 (d, 3 H, $J_{5,6}$ 6.5 Hz, CH_3), 1.92 (s, 3 H, NAc), 2.87 (s, 6 H, $N(CH_3)_2$), 4.36 (d, 2 H, $J_{1',2'}$ = $J_{1,2}$ 7 Hz, H-1′, H-1), 5.3 (d, 1 H, $J_{1'',2''}$ 3.7 Hz, H-1″), 7.25, 7.58, 8.19, 8.3, 8.55 (6 H, dansyl). LRMS: calc. for $C_{38}H_{59}O_{17}N_3S$: *m/z* 861.9; found 884.4 (*M* + Na^+).

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