A novel plant α4-fucosyltransferase (*Vaccinium myrtillus* L.) synthesises the Lewis[a] adhesion determinant

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Abstract We have partially characterised an α4-fucosyltransferase (α4-FucT) from *Vaccinium myrtillus*, which catalysed the biosynthesis of the Lewis[a] adhesion determinant. The enzyme was stable up to 50°C. The optimum pH was 7.0, both in the presence and in the absence of Mn^{2+}. The enzyme was inhibited by Mn^{2+} and Co^{2+}, and showed resistance towards inhibition with *N*-ethylmaleimide. It transferred fucose to *N*-acetylglucosamine in the type I Galβ3GlcNAc motif from oligosaccharides linked to a hydrophobic tail and glycoproteins (containing the type I motif). Sialylated oligosaccharides containing the type II Galβ4GlcNAc motif were not acceptors. The catalytic mechanism of the plant α4-FucT possibly involves a His residue, and it must have arisen by convergent evolution relative to its mammalian counterparts.

Key words: Plant complex glycan; Type I motif; Lewis[a]; Fucosyltransferase; *Vaccinium myrtillus*

1. Introduction

In plant glycoproteins two types of *N*-glycans are known: oligomannose glycans with the composition $Man_{5-9}GlcNAc_2$ and complex glycans with fewer mannose residues and additional monosaccharide residues: fucose (Fuc), xylose and galactose (Gal). Fuc residues have been found α3-linked to proximal GlcNAc [1,2], and to peripheral GlcNAc from secreted glycoproteins of plant cells in suspension cultures of *Vaccinium myrtillus* L. [3] and sycamore cells [4].

The Lewis[a] (Le[a]) determinant consists of a terminal Galβ3-(Fucα4)GlcNAc trisaccharide. It is synthesised by transferring a Fuc residue, in an α4 linkage, onto the *N*-acetylglucosamine (GlcNAc) residue from the type I chain (Galβ3GlcNAc), in a reaction catalysed by an α4-fucosyltransferase (α4-FucT). Le[a] has been previously found on cell-surface glycoconjugates from mammals, where it is involved in cell recognition and adhesion processes [5]. In plants, Le[a] is widely distributed and is detected on the cell surface [4], but it is not found in vacuolar glycoproteins [6]. This location may suggest some involvement of Le[a] in cell-to-cell recognition or interaction with plant pathogens.

Until now, three α4-FucT have been cloned: two of mammalian origin, FucT-III and FucT-V, and one from a bacterium, α3/4-FucT from the gastric pathogen *Helicobacter pylori* [7]. FucT-III has predominant α4-FucT activity with residual α3 activity, whereas FucT-V and the bacterial enzyme have mainly α3 activity.

In this paper we partially characterise the *V. myrtillus* α4-FucT, which synthesises the Le[a] previously identified by us [3]. The enzyme shows predominant α4-FucT activity and uses, as substrates, oligosaccharides linked to a hydrophobic tail and glycoproteins containing the type I determinant. Under standard conditions, the enzyme is inhibited by Mn^{2+} and Co^{2+} and shows resistance towards inhibition with *N*-ethylmaleimide (NEM). These results suggest the identification of a novel α4-FucT activity.

2. Materials and methods

2.1. Materials

Cell suspension cultures of *V. myrtillus* L. were maintained and subcultured every 9 days, as previously described [8].

Guanosine diphosphate (GDP) [^{14}C]fucose (287 mCi/mmol) and unlabelled GDP-Fuc were purchased from Amersham Pharmacia Biotech. The bovine serum asialofetuin and NEM were obtained from Sigma.

The type I acceptors, Galβ3GlcNAc-O-$(CH_2)_3$NHCO$(CH_2)_5$-NH-biotin (Galβ3GlcNAc-O-sp-biotin), Fucα2Galβ3GlcNAc-O-sp-biotin and NeuAcα2-3Galβ3GlcNAc-O-sp-biotin, and the type II acceptors, Galβ4GlcNAc-O-sp-biotin, Fucα2Galβ4GlcNAc-O-sp-biotin and NeuAcα2-3Galβ4GlcNAc-O-sp-biotin were purchased from Syntesome.

2.2. Microsomal fraction isolation

Nine-day-old cell suspension cultures were filtered through filter paper (Whatman, 91), cells were weighed and aggregates were split with an Ultraturrax (Ika, T8) four-fold, 30 s in homogenisation buffer (20 mM MES–NaOH, pH 6.8, 100 mM NaCl, 1 mM DTE, 1 μg/ml aprotinin, 1 mM EDTA). The cells were homogenised under pressure using a French press (APV Gaulin, 15 MR8TBA), two cycles at 8000 psi, and filtered trough a metallic net (pore diameter 150 μm). The homogenate was centrifuged at 1000×*g* for 10 min to remove cell debris, cell walls and unbroken cells. The supernatant was ultracentrifuged (Beckman, XL-100) at 100 000×*g*, for 60 min. The pellet, the microsomal fraction, was resuspended in extraction buffer (20 mM MES–NaOH, pH 6.8, 100 mM NaCl, 1 mM DTE, 1% Triton X-100, 25% glycerol) (0.1 ml/g fresh weight of cells). The resuspended pellet was extracted twice for 4 h followed by 16 h, and ultracentri-

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Abbreviations: Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Le[a], Lewis[a]; NEM, *N*-ethylmaleimide; α4-FucT, α4-fucosyltransferase

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fuged at 100 000×*g*, for 60 min. The α4-FucT activity was concentrated in this supernatant. All steps were performed at 4°C.

The microsomal fraction was stored at −80°C without loss of enzyme activity.

2.3. α4-FucT assay

The α4-FucT activity was determined using the following standard assay mixture: 1 μl of microsomal fraction, 0.35 mM of the acceptor substrate (Galβ3GlcNAc-O-sp-biotin), 1.7 μM GDP-[^{14}C]Fuc and 0.047 mM GDP-Fuc, 100 mM MOPS–NaOH, pH 7.0, 100 mM NaCl and 1% (v/v) Triton X-100, in a total volume of 12.5 μl. The effects of temperature and metals were studied in the same assay mixture. Temperature was studied in the range of 10–60°C. Divalent cations were tested at 20 mM concentration and Cl^- was the counterion used. The effect of pH was studied by adjusting the pH value of the assay mixture to final values in the range of 4.5–11.3. A range of increasing concentrations of NEM (0.001–10 mM) was added to the assay mixture in order to evaluate the effect of this sulphydryl-binding reagent on α4-FucT activity. The samples were left at 4°C for 45 min.

Incubations of 60 min at 37°C were performed and the reaction was stopped with cold water, with the product being separated from unincorporated label by reverse phase chromatography on a Sep-Pack C_{18} column according to Costa et al. [9]. The reaction rate varied directly with protein concentration, when the assay was performed with 50 mM MOPS, pH 7.5, in the presence of 20 mM $MnCl_2$, and was linear for at least 1 h.

Substrate specificity with small oligosaccharides linked to a hydrophobic tail was analysed using 0.35 mM of the acceptors Galβ3GlcNAc-O-sp-biotin, Fucα2Galβ3GlcNAc-O-sp-biotin, NeuAcα2-3Galβ3GlcNAc-O-sp-biotin, Galβ4GlcNAc-O-sp-biotin, Fucα2-Galβ4GlcNAc-O-sp-biotin or NeuAcα2-3Galβ4GlcNAc-O-sp-biotin. The glycoprotein asialofetuin, which contains type I structures in the terminal antenna of its *N*-glycans, was tested at 4 mg/ml. The reaction mixture was the same as above except that it contained 4.5 μM GDP-[^{14}C]Fuc and 0.045 mM GDP-Fuc. After 3 h of incubation, the reaction mixture was precipitated with 1% (w/v) cold tungstophosphoric acid in 0.5 M HCl. Radiolabelled protein was separated on Whatman GF/C membranes as previously described [9].

3. Results

3.1. Partial purification of α4-FucT from *V. myrtillus* cells

Cells in suspension were homogenised in a French press, and were almost totally broken as visualised by light microscopy. Since α4-FucT is a Golgi membrane protein, fractionation of the homogenate was performed by successive centrifugation in order to obtain an enriched microsomal fraction. The α4-FucT activity was monitored during the fractionation procedure (Fig. 1). It was observed that 24% of the initial α4-FucT activity was recovered in the 100 000×*g* pellet. This low recovery was due to enzyme inactivation during the fractionation procedure, which could not be overcome upon addition of a protease inhibitor cocktail. The enzyme was solubilised from the microsomal fraction with Triton X-100 in a two-step procedure, which was essential for increasing the extraction yield. A final 22% recovery of α4-FucT activity was achieved. This preparation was used for the enzyme assays described below.

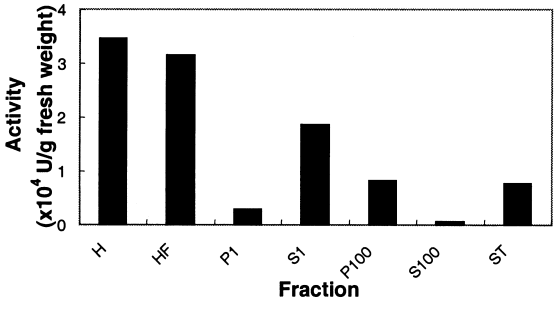

Fig. 1. Partial purification of α4-FucT from *V. myrtillus*. H, homogenate; HF, filtered homogenate; P1, pellet after 1000×*g* centrifugation; S1, post 1000×*g* supernatant; P100, pellet after 100 000×*g* centrifugation; S100, post 100 000×*g* supernatant; ST, Triton X-100 extract.

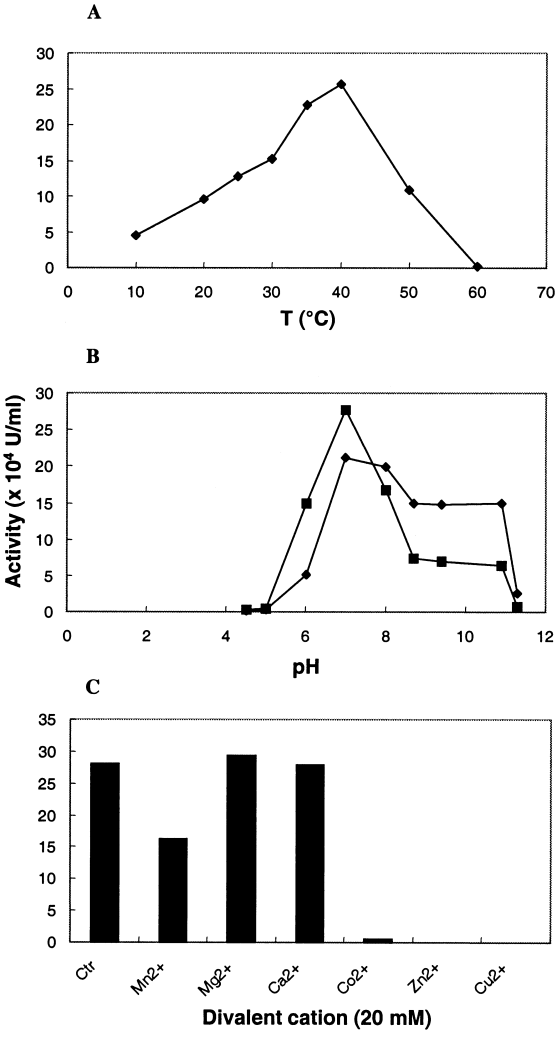

Fig. 2. Effects of temperature (A), pH (B) and divalent cations (C) on Fuc transfer to Galβ3GlcNAc-O-sp-biotin by α4-FucT from *V. myrtillus*. A: The temperature effect was tested within the range of temperatures of 10–60°C, under standard assay conditions. B: The pH effect was analysed under standard assay conditions using 100 mM MOPS buffer at different pH values, in the presence (♦) or absence (■) of 20 mM $MnCl_2$. C: The effects of divalent cations were determined under standard assay conditions with different divalent cations at a final concentration of 20 mM. Assays proceeded as described in Section 2.3. Data represent an average value of two series of independent experiments, where the maximum error estimated was 13%.

3.2. The biochemical properties of α4-FucT

After incubation of *V. myrtillus* α4-FucT at various temperatures (10, 20, 25, 30, 35, 40 and 60°C), it was observed that it had a maximal activity at 40°C, and it was rapidly inactivated above this value (Fig. 2A). A less sharp change in slope was observed between 25 and 30°C.

The enzyme activity as the function of pH was determined

in the presence or absence of Mn^{2+} (Fig. 2B). In both cases, below pH 5.0, there was no activity detected. Both in the presence and in the absence of Mn^{2+}, the optimum pH value was 7.0. In the presence of the cation, the enzyme showed a broader range of optimum pH between 7.0 and 8.0. In both cases, the activity of the enzyme was constant between pH 8.7 and 10.9 and decreased to residual levels at pH 11.3. The cation Mn^{2+} was an activator of α4-FucT at pH values above 8.0; below this value it was a mild inhibitor.

Tested with a range of divalent cations at 20 mM concentration, the α4-FucT was not affected by Mg^{2+} and Ca^{2+}, whereas the enzyme was completely inactivated by Zn^{2+} and Cu^{2+} and 40% and 98% inhibited by Mn^{2+} and Co^{2+}, respectively (Fig. 2C).

The activity of α4-FucT in the presence of increasing concentrations of the sulphydryl-binding reagent NEM was tested. The activity was reduced by only 30% at a NEM concentration of 10 mM. At this concentration, a NEM-sensitive FucT, e.g. human recombinant FucT-III from COS cells, was 85% inhibited [10]. On the other hand, a NEM-resistant bacterial α3-FucT from *H. pylori* was 34% inhibited at 15 mM NEM [11]. These results suggested that the *V. myrtillus* α4-FucT was resistant to NEM inhibition.

3.3. In vitro acceptor substrate specificity of α4-FucT

The substrate specificity of α4-FucT was investigated with a range of type I and type II oligosaccharide acceptors bearing a hydrophobic spacer arm conjugated to biotin (Table 1). The enzyme exhibited a preference for the transfer of Fuc to acceptors based on type I chains. Substitution of the terminal β-galactosyl residue of a type I acceptor (**1**, Table 1) with α2-linked Fuc (**2**, Table 1) slightly enhanced the activity (1.2-fold), but substitution with α2-3-linked sialic acid (**3**, Table 1) markedly decreased the recognition of the type I acceptor as the substrate (95% inhibition). The type II-based acceptors were extremely poor substrates for the enzyme, although a slight enhancement in the activity was observed with the substitution of the terminal disaccharide (**4,** Table 1) with α2-linked Fuc (**5**, Table 1). The type II acceptor substituted in the terminal β-galactosyl residue with α2-3-linked sialic acid (**6,** Table 1) was not an acceptor for the enzyme in our assay conditions.

Table 1
Acceptor specificity of α4-FucT from the microsomal fraction of *V. myrtillus* with low molecular weight oligosaccharide and glycoprotein substrates

Substrate[a]	Relative activity[b] (%)
1. Galβ3GlcNAc-O-sp-biotin	100 (100)[c]
2. Fucα2Galβ3GlcNaC-O-sp-biotin	118 (193)
3. NeuAcα2-3Galβ3GlcNAc-O-sp-biotin;	5.4 (57)
4. Galβ4GlcNAc-O-sp-biotin	0.4 (ND)
5. Fucα2Galβ4GlcNAc-O-sp-biotin	10.5 (9.3)
6. NeuAcα2-3Galβ4GlcNAc-O-sp-biotin	ND (ND)
7. Asialofetuin	7.3 (ND)

Data represent an average value of two series of independent experiments where the maximum error estimated was 7%.
[a]The acceptor substrate concentration was 0.35 mM, except for asialofetuin, which was tested at 4 mg/ml.
[b]Activity relative to Galβ3GlcNAc-sp-biotin which had an activity value of 2.3 mU/ml. ND, not detectable ($< 0.01\%$ relative activity).
[c]Values in parentheses refer to the full-length form of the human FucT-III purified from baby hamster kidney cells; 100% activity corresponded to 1.4 mU/ml (V. Sousa and J. Costa, unpublished results).

The enzyme showed activity towards the glycoprotein asialofetuin (**7**, Table 1), whose triantennary *N*-glycans contain the type I motif acceptor on one arm (35% of the total glycans) [9].

4. Discussion

In this study we have partially characterised a novel α4-FucT that catalyses the biosynthesis of Le^a type structures and that was previously identified in cellular extracts of *V. myrtillus* suspension cultures [3].

For characterisation studies, the α4-FucT activity from microsomal extracts, solubilised with Triton X-100, was measured using a radiometric assay. The rate of Fuc transfer was proportional to enzyme concentration, suggesting that no inhibitory substances were present in the microsomal fraction, capable of suppressing the α4-FucT activity.

The in vitro temperature dependence of α4-FucT activity showed that this plant enzyme was stable up to 50°C, and exhibited maximal activity between 35 and 40°C, the expected zone for mammalian FucTs. The source of this enzyme seems not to influence the susceptibility towards temperature variation.

The pH dependence of Fuc transfer was investigated both in the presence and in the absence of the divalent cation Mn^{2+}. The enzyme was active in the pH range 6.0–10.9 in both cases, with an optimum pH of 7.0, and with Mn^{2+} being an inhibitor below pH 8.0. Our previous observations showed that human FucT-III was active already at pH 4.0 in the absence of Mn^{2+}, which became an activator at pHs above 6.0 (A. Palma and J. Costa, unpublished results). The results obtained for FucT-III suggested the participation of an Asp residue, reported as an essential residue for activity [12] in the catalytic mechanism of the enzyme. The shift in activity of the plant α4-FucT towards higher pH values suggested that amino acid residues with pK_a values higher than Asp, such as His ($pK_a = 6.04$), might be involved in the reaction mechanism. The α4-FucT activity was tested in the presence of other divalent cations that were reported to influence the activity of FucTs [13–15]. Similarly to human FucT-III, total inactivation of the enzyme with Zn^{2+} and Cu^{2+} was observed. In contrast, the plant enzyme was inhibited with Mn^{2+} and Co^{2+}, but was not affected by Ca^{2+} and Mg^{2+}.

The α4-FucTs catalyse the transfer of Fuc from a GDP-β-L-Fuc donor to an oligosaccharide acceptor containing the type I structures in a α4 linkage. A general base is thought to assist in deprotonating the nucleophile hydroxyl group of the acceptor that attacks the C1 of the donor in an SN2-like mechanism and the reaction is Mn^{2+} ion-dependent [15]. The different specificity patterns for divalent cations obtained for α4-FucT and the non-dependence on Mn^{2+} for maximal activity support the idea of a different catalytic mechanism for this plant enzyme. A His residue is likely to be involved in the mechanism due to the pH profile observed (above pH 6.0), and to the inhibition by Co^{2+}, which specifically binds to His residues in certain proteins [16,17].

Another biochemical property used for FucT characterisation was its sensitivity to inactivation by sulphydryl group-modifying reagents such as NEM. The results obtained for this plant α4-FucT demonstrated that the enzyme activity was reduced by only 30% at a high concentration of the inhibitor (10 mM), in contrast to 85% inactivation at this con-

centration obtained for human FucT-III [10]. Previous studies on FucTs revealed that sensitivity to NEM is related to a conserved Cys residue that participates in GDP-Fuc binding while those involving different amino acids (FucT-IV has Ser, FucT-VII has Thr) are resistant to NEM inhibition [10]. The partial resistance obtained indicates that another analogous residue in the plant α4-FucT is involved in this function. Similar results were obtained for the cloned α3-FucT from *H. pylori* that was shown to contain a Tyr residue at this position [11].

Specificity studies with a range of low molecular weight oligosaccharide acceptors linked to a hydrophobic tail revealed that the type I acceptors were the most effective substrates for the plant enzyme. The presence of a Fuc substituent in α2 linkage to the terminal β-galactosyl residue slightly enhanced the efficiency of the enzyme towards type I acceptors. In contrast, the addition of sialic acid-linked α2-3 to the terminal β-galactosyl residue in type I structures strongly reduced the capacity of the oligosaccharide to function as an acceptor. When compared to the full-length FucT-III, this inhibition of plant α4-FucT activity was 10-fold higher (see Table 1). This was an expected difference since plants, in contrast to animals, do not have sialic acid residues. The corresponding type II acceptors were all poor substrates for the plant enzyme, although the capacity of the type II acceptor with α2-linked Fuc to be a substrate could be enhanced if the time of reaction was extended. These results reveal that the plant enzyme is preferentially an α4-FucT, exhibiting a residual α3 activity, similarly to human full-length FucT-III.

The striking feature of the plant α4-FucT is that the glycoprotein asialofetuin was a substrate for the enzyme, contrary to human full-length FucT-III (see Table 1). The different acceptor specificity pattern is possibly related to a distinct structure that allows the interaction of glycoproteins with the catalytic active site. The capacity of this plant enzyme to use glycoproteins as substrates is in agreement with our previous identification of the Le^a determinant in a secreted peroxidase from *V. myrtillus* [3].

The differences in enzymatic mechanism and substrate specificity suggest that the plant α4-FucT results from convergent evolution relative to mammalian α4-FucTs.

The plant α4-FucT can be used for the in vitro synthesis of glycomimetics, potential inhibitors of the adhesion processes associated with metastasis formation or pathogenic events where the Le^a determinant, and derivatives, mediate the recognition (e.g. *H. pylori* infection of stomach cells).

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