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β-1,4-Galactosyltransferase-catalyzed glycosylation of sugar derivatives: Modulation of the enzyme activity by α-lactalbumin, immobilization and solvent tolerance

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Abstract

The influence of different parameters on the activity of the β-1,4-galactosyltransferase (β-1,4-GalT) from bovine milk has been investigated using various acceptor and donor substrates. It was found that the "specifier" protein α-lactalbumin (α-LA), which interacts with β-1,4-GalT forming the lactose synthase (LS) complex, is not necessary when the acceptors are different glucopyranosides, and, in some cases, it can even have an inhibitory effect, like with the complex glucosides ginsenoside Rg_1 (**1**) and colchicoside (**2**). By optimization of the reaction conditions, the galactosylated and glucosylated derivatives of **2** were prepared, using UDP-Gal and UDP-Glc as sugar donors, respectively, and characterized. Moreover, β-1,4-GalT was covalently immobilized on Eupergit C 250 L in the absence of α-LA, and the synthetic performances of this immobilized biocatalyst were evaluated. Finally, the best organic cosolvents to be used both with β-1,4-GalT and the LS complex were identified.

Keywords: β-1,4-Galactosyltransferase; Enzymatic galactosylation; α-Lactalbumin; Immobilization; Organic solvent

1. Introduction

The glycosyltransferases from the Leloir pathway, responsible for the synthesis of most cell-surface glycoforms in mammalian systems, have been exploited as viable alternatives in the preparation of oligosaccharides [1]. These enzymes are highly regio- and stereospecific with respect to the formed glycosidic linkages and make use of unprotected sugar precursors, thus avoiding tedious chemical elaborations of these molecules. Several recombinant glycosyltransferases are now available from well-established fermentation processes, and, employing metabolic pathway engineering, large-scale production systems for most of the nucleoside mono- and diphosphate sugar donors have also been recently developed [2,3].

The β-1,4-galactosyltransferase (β-1,4-GalT, EC 2.4.1.22) [4] from bovine milk is one of the most extensively studied enzymes of this group, having been deeply investigated for its substrate specificity and synthetic performances. Moreover, it is the first mammalian glycosyltransferase whose three-dimensional structure has been elucidated [5].

β-1,4-GalT catalyzes the transfer of galactose (Gal) from UDP-Gal to the OH at the C-4 position of *N*-acetylglucosamine (GlcNAc) acceptors to give *N*-acetyllactosamine (LacNAc). When the enzyme interacts with α-lactalbumin (α-LA), a mammary gland-specific Ca^{2+} ion-binding protein, a lactose synthase (LS) complex is formed, which transfers Gal to glucose (Glc), producing lactose (Lac) [6,7].

Numerous other acceptor substrates for this enzyme have been described in the literature, e.g., 2-deoxyglucose, D-xylose, 5-thioglucose, *N*-acetylmuramic acid and *myo*-inositol [8,9]. Moreover, modifications at the OH-3 or OH-6 positions of the acceptor GlcNAc are usually well-tolerated: for example, 6-*O*-fucosylated and sialylated derivatives served as acceptors, as well as 3-*O*-methyl GlcNAc, 3-deoxy-GlcNAc, butyl 3-*O*-allyl-β-D-GlcNAc and 3-oxo-GlcNAc [10–12]. Additionally, several GlcNAc and Glc derivatives were employed as acceptor substrates for the synthesis of complex oligosaccharides, both in solution [13] and in solid phase [14,15]. Finally, β-1,4-GalT-

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catalyzed galactosylations have been recently exploited for the modification of pharmacologically interesting glycosides, e.g., alkaloid glycosides [16,17], stevioside and steviolbioside [18], the coumarinic glycoside fraxin [17] and different ginsenosides [19,20].

Concerning the donor specificity, β-1,4-GalT shows a strong preference for UDP-Gal, but this is not an absolute requirement. In fact, it has been reported that the transfer of Glc, 2-deoxy-Glc, arabinose and *N*-acetylgalactosamine (GalNAc) from the corresponding UDP-derivatives, is also possible, albeit with low rates (0.3–5%) in comparison with Gal transfer [21–23].

Several crystal structures of the complex between β-1,4-GalT and α-LA in the presence of various substrates have been recently determined, leading to a better understanding of the enzyme mechanism and of the modulation of the acceptor specificity by α-LA in the LS complex [24]. However, the effect of α-LA on the acceptor specificity of β-1,4-GalT when using substrates differing from the natural ones has not been specifically investigated yet. We have focused our endeavor to this problem and here we report on the effect of α-lactalbumin on the reaction rates with three different sugar donors (UDP-Gal, UDP-Glc and UDP-GlcNAc) and several acceptor substrates. Moreover, the effect of various water-miscible organic cosolvents on β-1,4-GalT and LS activities has been investigated. These results have been exploited for new β-1,4-GalT synthetic applications, and, as an example, different glycosylated derivatives of the glucoside colchicoside have been isolated and characterized.

2. Experimental

2.1. General experimental

α-Lactalbumin from bovine milk (type I), phosphoenolpyruvic acid monopotassium salt (PEP), pyruvate kinase (PK, EC 2.7.1.40, type III from rabbit muscle), lactate dehydrogenase (LDH, EC 1.1.1.27, type XI from rabbit muscle), alkaline phosphatase (AP, EC 3.1.3.1, type VII from bovine intestinal mucosa), *N*-acetylglucosamine (GlcNAc), β-nicotinamide adenine dinucleotide, reduced form (NADH) and $MnCl_2{\cdot}4H_2O$ were from Sigma. β-1,4-Galactosyltransferase from bovine milk (β-1,4-GalT, EC 2.4.1.22) and uridine 5′-diphosphogalactose (UDP-galactose, UDP-Gal) were from Fluka (β-1,4-galactosyltransferase KIT, no. 59505). Methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, phenyl β-D-glucopyranoside, maltose and cellobiose were from Aldrich. Uridine 5′-diphosphoglucose (UDP-glucose, UDP-Glc) was from Pro. Bio. Sint. S.p.A (Varese, Italy). Uridine 5′-diphospho-*N*-acetylglucosamine (UDP-*N*-acetylglucosamine, UDP-GlcNAc) was a gift from Dr. Endo, Kyowa Hakko Kogyo Co., Japan. Ginsenoside Rg_1 (**1**) and colchicoside (**2**) were a gift from Prof. Danieli, Milano University. Eupergit C 250 L was from Rohm Pharma GmbH (Darmstadt, Germany). All the other chemicals were of analytical grade.

Thin-layer chromatography (TLC): analytical (5 cm × 10 cm) and preparative (20 cm × 20 cm) precoated silica gel 60 F_{254} plates (Merck). Substrates and products were visualized at 254 nm and by plates treatment with the molybdate reagent ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 concentrated, 62 ml; made up to 1 l of deionized water). Flash chromatography: silica gel 60 (70–230 mesh, Merck). HPLC analyses were carried out using a Jasco 880-PU pump equipped with a Jasco 870-UV detector and a Hewlett-Packard HP-3395 integrator. Enzymatic activities were monitored using a Jasco V-530 UV/vis spectrophotometer. ^1H NMR and ^{13}C NMR spectra at 300 and 75.2 MHz were recorded in Me_2SO-d_6 on a Bruker AC-300. FAB-MS spectra were recorded using a VG 7070 EQ-HF spectrometer equipped with its own source, operating at 8 keV with xenon gas and in diethanolamine as matrix. Electrospray mass spectrometry (ESI-MS) spectra were recorded with a LCQ^{TM} quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a Nano-ESI source (Protana, Odense, Denmark). Due to the unavoidable presence of Na^+ ions during sample preparation and the high alkali-cation affinity of oligosaccharides, all positively charged ions produced were ionized with Na^+.

2.2. Spectrophotometric assay of β-1,4-GalT activity

β-1,4-GalT activity was assayed spectrophotometrically with sugar acceptors in the presence and in the absence of α-LA (0.2 mg/ml), at 30 °C, in the following assay solution: glycyl glycine buffer, pH 8.4 (50 mM), KCl (50 mM), $MgCl_2$ (11 mM), $MnCl_2{\cdot}4H_2O$ (5 mM), PEP (1.3 mM), NADH (0.25 mM), UDP-sugar (UDP-Gal or UDP-Glc or UDP-GlcNAc, 0.32 mM), sugar acceptor (18 mM), PK (6.3 U/ml), LDH (9 U/ml), β-1,4-GalT (6.8×10^{-3} to 6.8×10^{-1} U/ml, depending on the reaction rate). The assay was started by addition of the sugar acceptor and consumption of NADH was checked continuously at 340 nm. One unit corresponds to the amount of enzyme which transfers 1 μmol of galactose from UDP-Gal to D-glucose per minute at pH 8.4 and 30 °C in the presence of α-LA. Freshly prepared assay solutions were tested by performing a reference reaction using Glc as an acceptor and UDP-Gal as a donor, in the presence of α-LA. The rate of the reference reaction was taken as 100% for comparison with the reactions performed in the presence of different acceptor and/or donor substrates (with or without α-LA).

2.3. Effect of α-lactalbumin on the galactosylation of colchicoside (*2*)

β-1,4-GalT (0.1 U) and alkaline phosphatase (5 U) were dissolved in 0.5 ml of 50 mM Tris/HCl buffer, pH 7.4, containing colchicoside (**2**, 3.5 mg, 13 mM), 1.5 equivalent of UDP-Gal (6 mg, 19.5 mM) and $MnCl_2{\cdot}4H_2O$ (10 mM). The reaction mixture was divided in two aliquots (250 μl each) and to one of them α-lactalbumin (4 mg/ml) was added. Both reactions were incubated at 37 °C for 24 h and checked at scheduled times by HPLC analysis (reverse-phase analytical column PurospherSTAR RP-18e, 5 μm, 4 mm × 125 mm, Merck; elution system: H_2O–CH_3CN, 92:8, 0.05%, v/v, CF_3COOH; flow

rate: 0.75 ml/min; UV detector: 254 nm; retention times: (**2**), 15.0 min; galactosyl-colchicoside (**3**), 12.7 min).

2.4. Immobilization of β-1,4-GalT

β-1,4-GalT (15 mg) and alkaline phosphatase (5 U) were dissolved in 4.95 ml of 1 M potassium phosphate buffer, pH 7.0. An aliquot of this enzyme solution (200 μl) was immediately checked for β-1,4-GalT activity, using colchicoside (**2**) as a substrate under the previously described conditions (total initial activity: 0.715 U), and stored for further comparison with the immobilized enzyme. Eupergit C 250 L (825 mg) was added to the remaining enzyme solution (4.8 ml) and the mixture was incubated at 4 °C overnight.

The resin was washed with 50 mM Tris/HCl buffer, pH 7.4, and the residual epoxy groups were saturated by incubation with 0.3 M ethanolamine in 1.2 M potassium phosphate buffer, pH 7.0, at 4 °C for 5 h. After this treatment, the immobilized biocatalyst (5 g wet weight) was further washed with 50 mM Tris/HCl buffer, pH 7.4, assayed for activity on **2** (recovered activity: 0.03 U, 4% yield), and stored at 4 °C.

2.5. Galactosylation of GlcNAc, ginsenoside Rg_1 (**1**) and colchicoside (**2**) with immobilized β-1,4-GalT

Immobilized β-1,4-GalT was used for the galactosylation of different sugar acceptors, e.g., GlcNAc, ginsenoside Rg_1 (**1**) and colchicoside (**2**). Immobilized β-1,4-GalT (0.5 g wet weight) was added to a solution of UDP-Gal (19.5 mM) and sugar acceptor (13 mM) in 0.5 ml of 50 mM Tris/HCl buffer, pH 7.4, containing $MnCl_2{\cdot}4H_2O$ (10 mM). The reactions were incubated at 37 °C and 150 rpm for 24 h and monitored by TLC (AcOEt–MeOH–H_2O, 8:4:1). The products formed were compared by TLC with authentic samples of *N*-acetyllactosamine, mono- and digalactosylated Rg_1 and galactosylated colchicoside.

2.6. Activity and stability of the immobilized β-1,4-GalT

UDP-Gal (6 mg, 19.5 mM) and colchicoside (**2**, 3.5 mg, 13 mM) were dissolved in 1.5 ml of 50 mM Tris/HCl buffer, pH 7.4 containing $MnCl_2{\cdot}4H_2O$ (10 mM). Immobilized β-1,4-GalT (0.5 g wet weight) was added and the reaction was incubated at 37 °C and 150 rpm. Samples were taken at scheduled times and the transfer of Gal from UDP-Gal to **2** was evaluated by HPLC analyses. After 24 h, the immobilized enzyme was recovered, washed extensively with 50 mM Tris/HCl buffer, pH 7.4, and reused in a new conversion cycle by addition of fresh substrate solution. Following this protocol, the immobilized biocatalyst was reused for 13 consecutive cycles (24 h each).

Storage stability was assayed performing the galactosylation of **2** under the above-mentioned conditions with an aliquot of immobilized enzyme stored at 4 °C for 5 weeks. No significant change of the initial reaction rate and of the conversion after 24 h was observed in comparison with the performances of the freshly immobilized enzyme.

2.7. TLC-evaluation of β-1,4-GalT activity with different donors and acceptors

The transfer of Glc and GlcNAc from the corresponding UDP-sugars to different sugar acceptors (GlcNAc, phenyl β-D-glucopyranoside, ginsenoside Rg_1 (**1**) and colchicoside (**2**)), catalyzed by β-1,4-GalT in the presence or in the absence of α-LA, was screened in analytical-scale reactions.

β-1,4-GalT (0.2 U) and alkaline phosphatase (5 U) were dissolved in 0.5 ml of 50 mM Tris/HCl buffer, pH 7.4, containing sugar acceptor (10 mM), 1.5 equivalent of UDP-sugar (15 mM) and $MnCl_2{\cdot}4H_2O$ (10 mM). This reaction mixture was divided in two aliquots (250 μl each) and to one of them α-lactalbumin (4 mg/ml) was added. Both reactions were incubated at 37 °C for 24 h and monitored by TLC (AcOEt–MeOH–H_2O, 8:4:1, or 10:3:0.5 for the reactions with the ginsenoside Rg_1).

2.8. Preparative synthesis of glycosylated derivatives of colchicoside (**2**)

2.8.1. Galactosyl-colchicoside (**3**)

Immobilized β-1,4-GalT (1.5 g wet weight) was added to 5 ml of 50 mM Tris/HCl buffer, pH 7.4, containing $MnCl_2{\cdot}4H_2O$ (10 mM), UDP-Gal (125 mg, 41 mM) and **2** (24.8 mg, 9.1 mM). The reaction was incubated at 37 °C and 150 rpm and monitored by TLC (AcOEt–MeOH–H_2O, 8:4:1) and HPLC analyses. The pH of the reaction was adjusted daily by dropwise addition of 0.5 M NaOH. After 5 days, the immobilized enzyme was filtered off and the reaction mixture was separated by flash chromatography (AcOEt–MeOH–H_2O, 8:4:1), to give 3.9 mg of **3** (11.8% yield). NMR and MS data for **3** were identical to those previously reported [17]. ^{1}H NMR (Me_2SO-d_6, 80 °C) selected data, δ: 8.35 (d, 1H, J = 7.5 Hz, NHCO), 7.18 (s, 1H, H-8), 7.12 (d, 1H, J = 9.5 Hz, H-12), 7.01 (d, 1H, J = 9.5 Hz, H-11), 6.88 (s, 1H, H-4), 5.01 (d, 1H, J = 7.5 Hz, H-1′), 4.39 (dt, 1H, J_1 = 7 Hz, J_2 = 7.5 Hz, H-7), 4.33 (d, 1H, J = 7.2 Hz, H-1″), 3.90, 3.88 and 3.60 (s, 3H each, CH_3O), 1.88 (s, 3H, CH_3CO). ^{13}C NMR (Me_2SO-d_6), δ: 178.4 (C-9), 169.2 (NHCO), 163.9 (C-10), 151.2, 151.0 and 150.7 (C-7_a, C-3 and C-1), 141.5 (C-2), 135.5 (C-12_a), 135.0 (C-12), 134.3 (C-4_a), 130.5 (C-8), 127.0 (C-1_a), 112.6 (C-11), 111.3 (C-4), 104.0 (C-1″), 100.3 (C-1′), 80.3 (C-4′), 75.7 (C-5″), 75.4 and 75.2 (C-5′ and C-3′), 73.3 (C-2′), 73.2 (C-3″), 70.8 (C-2″), 68.3 (C-4″), 61.3 (CH_3O), 61.2 (CH_3O), 60.6 (C-6″), 60.3 (C-6′), 56.4 (CH_3O), 51.6 (C-7), 35.8 (C-5), 29.4 (C-6), 22.6 (CH_3). FAB-MS: 709 (*M*-H, 8), 677 (10), 547 (*M*-Gal-H, 60), 531 (23), 515 (22), 385 (*M*-Gal-Glc-H, 100), 371 (52).

2.8.2. Glucosyl-colchicoside (**4**)

β-1,4-GalT (3 mg, 3 U) was added to 10 ml of 50 mM Tris/HCl buffer, pH 7.4, containing alkaline phosphatase (5 U), $MnCl_2{\cdot}4H_2O$ (10 mM), UDP-Glc (390 mg, 64 mM) and **2** (70 mg, 12.8 mM). The reaction was incubated at 30 °C and monitored by TLC (AcOEt–MeOH–H_2O, 8:4:1) and HPLC analyses. The pH of the reaction was adjusted daily by dropwise addition of 0.5 M NaOH. After 4 days, the reaction mixture was freeze-dried and unreacted UDP-Glc was removed

by MeOH extraction. The residue (273 mg) was dissolved in 0.5 ml of dimethylsulfoxide and separated by preparative TLC (AcOEt–MeOH–H_2O, 8:4:1), to give 5 mg of **4** (5.4% yield). ^{1}H NMR (Me_2SO-d_6, 80 °C) selected data, δ: 7.14 (s, 1H, H-8), 7.12 (d, 1H, J = 11.2 Hz, H-12), 7.04 (d, 1H, J = 11.2 Hz, H-11), 6.87 (s, 1H, H-4), 5.01 (d, 1H, J = 7.5 Hz, H-1′), 4.33 (dt, 1H, J_1 = 7 Hz, J_2 = 7.5 Hz, H-7), 4.26 (d, 1H, J = 6.8 Hz, H-1″), 3.88, 3.84 and 3.57 (s, 3H each, CH_3O), 1.86 (s, 3H, CH_3CO). ^{13}C NMR (Me_2SO-d_6), δ: 178.5 (C-9), 169.0 (NHCO), 164.0 (C-10), 151.1, 151.0 and 150.6 (C-7_a, C-3 and C-1), 141.9 (C-2), 135.6 (C-12_a), 134.9 (C-12), 134.4 (C-4_a), 130.8 (C-8), 127.3 (C-1_a), 112.6 (C-11), 111.7 (C-4), 104.2 (C-1″), 100.8 (C-1′), 80.6 (C-4′), 76.0 (C-5″), 75.7 and 75.6 (C-5′ and C-3′), 73.7 (C-2′), 73.6 (C-3″), 71.0 (C-2″), 68.5 (C-4″), 61.3 (CH_3O), 61.2 (CH_3O), 60.8 (C-6″), 60.2 (C-6′), 56.5 (CH_3O), 51.7 (C-7), 36.0 (C-5), 29.4 (C-6), 22.8 (CH_3). ESI-MS: 732.25075 $[M+Na]^+$ (theoretic: 732.24741).

2.9. Screening of β-1,4-GalT and LS activity in the presence of organic cosolvents

The screening of the effect of a fixed concentration (20%, v/v) of several water-miscible organic cosolvents on β-1,4-GalT and LS activity was performed by spectrophotometric determinations using the same assay conditions previously described. The assays were carried out in the presence or in the absence of α-LA, using UDP-Gal as a sugar donor and Glc and GlcNAc as acceptors, respectively. The solvent was added immediately before the sugar acceptor and consumption of NADH was checked continuously at 340 nm. The reactions rates in the presence of cosolvents were compared with those observed in reference reactions carried out in the absence of cosolvents (100%).

3. Results and discussion

3.1. Effect of α-lactalbumin on the galactosylation of different sugar acceptors

The β-1,4-GalT from bovine milk tolerates substitutions at different positions of the sugar acceptor, provided that the equatorial C-4 hydroxyl is always present.

Although the ability of α-LA to modulate the specificity of β-1,4-GalT for the substrate glucose in the LS complex is well-known, no information is available on the effect of this "specifier" protein on the galactosylation of complex glucosides.

The effect of α-LA on β-1,4-GalT activity using Glc, GlcNAc and different α- and β-glucopyranosides as acceptors and UDP-Gal as sugar donor, was first assayed spectrophotometrically by measuring UDP formation (Table 1, columns 2 and 3). The complex glucoside ginsenoside Rg_1 (**1**, Scheme 1), which was previously shown to be a good substrate for β-1,4-GalT [19], was chosen to highlight the influence of a bulky and hydrophobic aglycon on the reaction rate in comparison with glucopyranosides linked to smaller aglycones, such as methyl and phenyl, or to hydrophilic groups, such as another sugar.

Concerning mono- and disaccharides and the methyl glucopyranosides, our investigations confirmed the results previously reported in the literature [6]. Lactose synthesis was achieved only in the presence of α-LA (Table 1, line 1), whereas this protein showed a strong inhibitory effect on LacNAc production (line 2). The relative rates of the reactions performed using α- or β-methyl glucopyranosides and α-1,4- or β-1,4-linked disaccharides were significantly lower than those determined using Glc as an acceptor and were only slightly affected by the presence of α-LA.

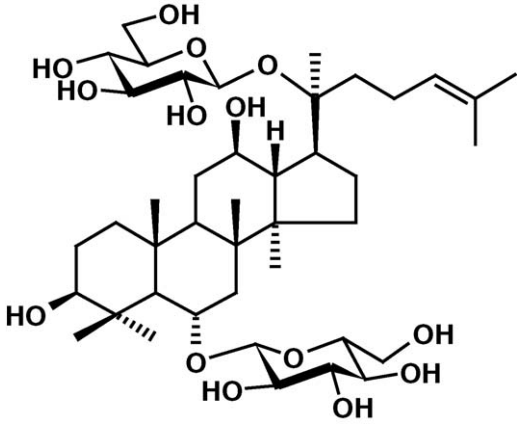
Table 1
Effect of α-LA on Gal, Glc and GlcNAc transfer with different sugar acceptors catalyzed by bovine milk β-1,4-GalT[a]

Acceptor	Percentage					
	UDP-Gal		UDP-Glc		UDP-GlcNAc	
	+α-LA	−α-LA	+α-LA	−α-LA	+α-LA	−α-LA
Glc	100	0	0.09	0.16	0.01	0.02
GlcNAc	27	82	0.06	0.06	0.04	0.03
α-Methyl Glc	0.05	0.03	0.03	0.02	0.03	0.02
Maltose	0.16	0.16	0.02	0.02	0.01	0.02
β-Methyl Glc	0.03	0.04	0.03	0.03	0.05	0.07
β-Phenyl Glc	0.60	0.60	0.12	0.10	0.03	0.06
Cellobiose	0.13	0.15	0.40	0.12	0.23	0.16
Ginsenoside Rg_1 (**1**)	58	74	0.50	0.70	0.08	0.26

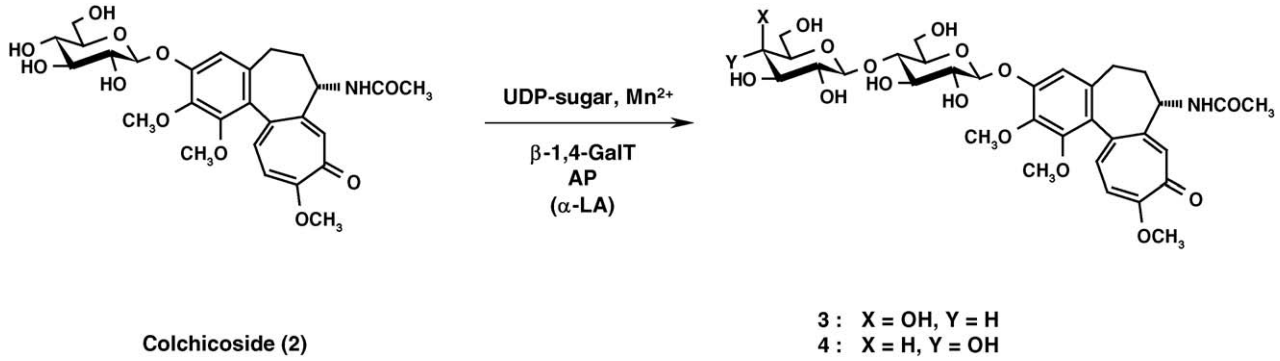
Relative initial rates are expressed as percentage of the lactose synthase reaction rate (first line of column 2).
[a] For details, see Section 2.

The presence of a more hydrophobic aglycon on the glucosidic acceptor seemed to positively affect β-1,4-GalT activity. Phenyl β-glucopyranoside showed to be a better acceptor than the corresponding β-methyl derivative, both in the presence and in the absence of α-LA, although the reaction rates were, in both cases, less than 1% of that observed for lactose synthesis.

Surprisingly, a significant improvement of the reaction rate was observed using **1** as an acceptor. Moreover, we found that, despite the strict requirement of α-LA for the lactose synthase activity (K_m for Glc is ∼2 M in the absence of α-LA, but is reduced by 1000-fold if α-LA is present [6]), not only this protein was not necessary with **1**, but it also had a negative effect on the reaction rate.



Scheme 1. Ginsenoside Rg_1 (**1**).



Scheme 2. Glycosylation of colchicoside (**2**) catalyzed by β-1,4-GalT in the presence of UDP-Gal or UDP-Glc.

To investigate further this phenomenon, we chose a different β-glucopyranosidic derivative, the alkaloid colchicoside (**2**, Scheme 2), which, similarly to **1**, possesses a bulky and hydrophobic aglycon.

Galactosylation of **2** catalyzed by β-1,4-GalT was performed either in the presence or in the absence of α-LA and, at scheduled times, the transformation of the acceptor substrate into the corresponding galactosylated derivative (**3**, Scheme 2) was monitored by HPLC (Fig. 1). Alkaline phosphatase was added to both reactions in order to reduce the effects of UDP inhibition. In the presence of α-LA, a strong inhibitory effect was observed: the initial reaction rate was reduced (about four times) and, after 20 h, the observed conversion was only about 20% instead of 50%.

A possible explanation of this inhibitory effect comes from the crystal structure analyses of the LS complex [25] and by previous structural and kinetic studies of β-1,4-GalT interaction with oligosaccharidic substrates [26]. In fact, it has been shown that the interactions between α-LA and β-1,4-GalT are primarily hydrophobic and that α-LA binds to β-1,4-GalT at the same site occupied by the non-reducing ends of oligosaccharide acceptors. Moreover, α-LA acts as a competitive inhibitor when chitobiose or other GlcNAc containing oligosaccharides are used as acceptor substrates [27]. Taking into consideration

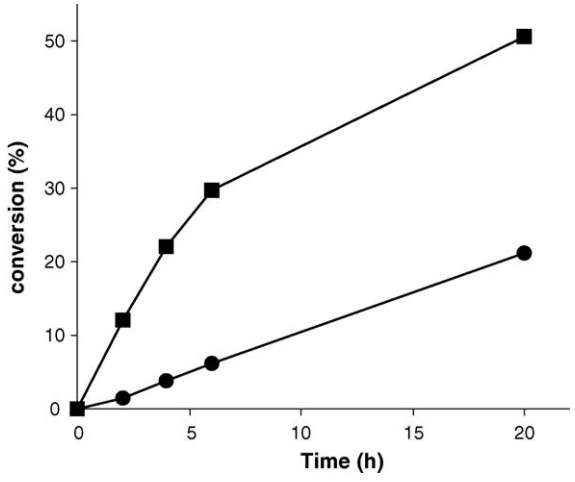


Fig. 1. Time course of the galactosylation of colchicoside catalyzed by β-1,4-GalT in the presence (●) or in the absence (■) of α-LA.

these data and our experimental results, we can hypothesize the binding of the hydrophobic aglycon of **2** (and, presumably, also of other similar β-glucopyranosides) at the same site that can be occupied by α-LA. These hydrophobic interactions could stabilize these unnatural substrates in their binding to β-1,4-GalT, thus making them very good targets for this enzyme, but, at the same time, α-LA might act as a "competitor" for these compounds.

Although the negative effect of α-LA addition on colchicoside galactosylation was quite clear, the previous experiments did not completely demonstrate that α-LA was not necessary for the reaction. In fact, it has been estimated that α-LA can be present as a contaminant (∼5% of total proteins) of the commercial bovine milk β-1,4-GalT preparations [28]. This contaminant α-LA was clearly not sufficient to activate the lactose synthase activity (Table 1), but, in principle, it was not possible to exclude its influence on the galactosylation of **2**.

In order to demonstrate that colchicoside could indeed be accepted as a substrate by β-1,4-GalT even without the participation of α-LA, the enzyme preparation was covalently immobilized on Eupergit C 250 L. In fact, it was shown by sedimentation analysis [29] and chemical cross-linking experiments [30] that α-LA and β-1,4-GalT interact only in the presence of substrate molecules. Moreover, the recent analysis of several crystal structures of the complex of β-1,4-GalT with α-LA in the presence of various substrates has shown that, upon binding of either the sugar acceptors or UDP-Gal and Mn^{2+}, β-1,4-GalT undergoes large conformational changes that create the α-LA-binding site and enable the formation of the LS complex [25]. Therefore, the covalent immobilization of β-1,4-GalT and of the (possible) contaminant α-LA in the absence of substrates is expected to physically separate these two proteins, obviously making their interaction impossible even by subsequent substrate addition.

First, the ability of the immobilized β-1,4-GalT to perform the galactosylation of different acceptor substrates, specifically GlcNAc and the glucosides **1** and **2**, in the absence of α-LA was tested on an analytical scale. TLC control of the reactions showed that all these substrates were satisfactorily converted into the expected products. Subsequently, the galactosylation of **2** catalyzed by the immobilized β-1,4-GalT was scaled up to a mg-scale and the reaction product (**3**) was recovered and characterized, thus confirming that the presence of α-LA was

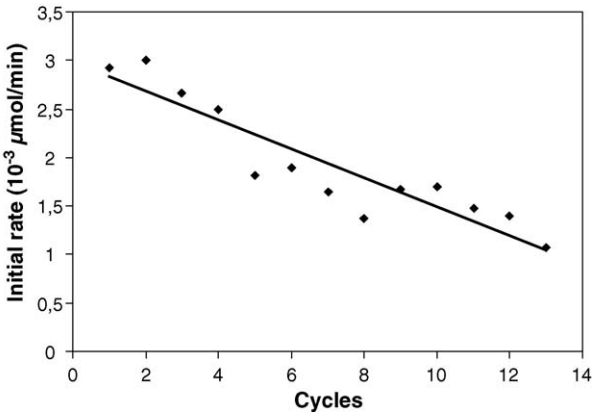


Fig. 2. Initial reaction rates of the galactosylation of **2** into **3** catalyzed by immobilized β-1,4-GalT.

not needed and that the reaction was actually catalyzed by β-1,4-GalT.

3.2. Synthetic performances of immobilized β-1,4-GalT

Despite the general interest for glycosyltransferases synthetic applications, only a few examples of covalent immobilization of β-1,4-GalT can be found in the literature [11,31,32]. Therefore, the synthetic performances of this biocatalyst in the galactosylation of **2** were evaluated by recycling the immobilized enzyme. The reaction was repeated for 13 cycles (24 h each), and the initial reaction rate of each cycle was determined by HPLC analyses of samples taken at scheduled times. Although a slight constant decrease of β-1,4-GalT activity was observed, at the end of this extensive use the immobilized enzyme still showed about 40% of the initial activity towards **2** (Fig. 2). Additional data are reported in Fig. 3, which shows the degrees of conversion of **2** into the product **3** after 8 h.

Finally, we evaluated the storage stability of the immobilized preparation by using an aliquot of immobilized enzyme stored at 4 °C for 5 weeks. The initial reaction rates and the degrees of conversion after 24 h were similar to those obtained using a freshly prepared immobilized biocatalyst.

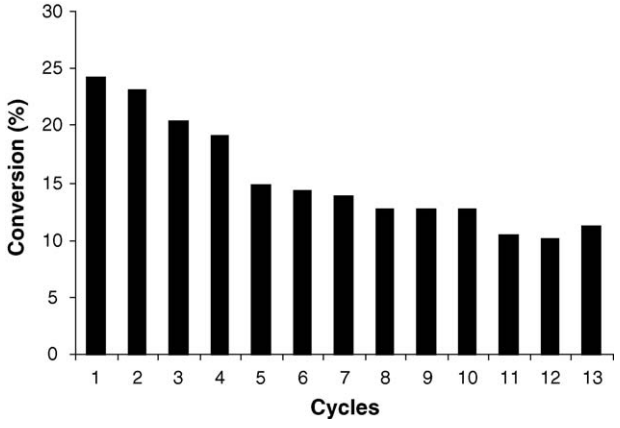


Fig. 3. Degrees of conversion after 8 h of **2** into **3** catalyzed by immobilized β-1,4-GalT.

3.3. Effect of α-lactalbumin on the donor specificity of bovine milk β-1,4-GalT

The donor specificity of Leloir pathway glycosyltransferases is typically restricted to one specific sugar-nucleotide, i.e., UDP-Gal in the case of β-1,4-GalT. Nevertheless, it has been reported that transfer of different UDP-sugars is possible, albeit at low rates compared with the natural reaction. In some cases, such low rates proved to be sufficient for the synthesis of preparative amounts of oligosaccharide and glycoconjugate derivatives. For example, using UDP-Glc as a donor, the isolation of β-glucosylated derivatives of **1** was reported [19], as well as the glucosylation of the ergot alkaloid elymoclavine and of different chitooligomers [16,33].

The effect of α-LA on the rate of transfer of Glc and GlcNAc from the corresponding sugar-nucleotides was investigated using different acceptors. Initially, spectrophotometric assays showed a strong decrease of the reaction rates when using these donors, ranging from 0.01 to 0.7% of that of the LS reaction (Table 1, columns 4–7). No significant change of the initial rates was observed in the presence of α-LA, besides a slight inhibitory effect on the glycosylation of the ginsenoside **1**.

Moreover, in order to establish the feasibility of the enzymatic glycosylation of different acceptors using UDP-Glc and UDP-GlcNAc as sugar donors, several reactions were screened on an analytical scale in the presence and in the absence of α-LA (see Section 2 for details). TLC control showed the formation of glycosylated derivatives only when using the glucopyranosides **1** and **2** as acceptors. In the case of **2**, a remarkable inhibitory effect of α-LA was observed, as previously described also for the galactosylation reaction.

Although the reaction rates when using these sugar-nucleotides as donors were significantly lower than those obtained with UDP-Gal, we succeeded in the preparation of the glucosylated derivative of **2** (**4**, Scheme 2) in the absence of α-LA, by scaling-up the reaction and recovering of the product, albeit in low yields, by preparative TLC.

3.4. Evaluation of β-1,4-GalT or LS activity in the presence of different organic cosolvents

The general exploitation of β-1,4-GalT for the modification of natural glycosides is quite often hampered by the low solubility of these substrates, due to the hydrophobic nature of their aglycons. The use of organic cosolvents might overcome this limitation.

We have previously investigated on the effect of various water-miscible organic cosolvents on the activity and stability of β-1,4-GalT in the presence of α-LA [17,34]. As a final step of the present work, we compared the influence of a fixed concentration (20%, v/v) of several cosolvents on enzyme activity in the presence or in the absence of α-LA, using UDP-Gal as a sugar donor and Glc and GlcNAc as acceptors, respectively. The reactions rates were determined by spectrophotometric assays and compared with those observed in the absence of cosolvents (Table 2). Some solvents, e.g., methanol and ethanol, were very well-tolerated, whereas some others, e.g., acetonitrile,

Table 2
Influence of different cosolvents (20%, v/v) on β-1,4-GalT activity in the presence (acceptor: Glc) and in the absence (acceptor: GlcNAc) of α-LA[a]

Cosolvent	Relative initial rates	
	Acceptor: Glc (+α-LA)	Acceptor: GlcNAc (−α-LA)
Blank	100	100
Ethanol	96	58
Methanol	117	103
Acetone	41	63
Acetonitrile	0	0
Tetrahydrofuran	0	0
N,*N*-Dimethylformamide	10	16
Dimethylsulfoxide	57	38
Dioxane	12	91
N-Methyl pyrrolidone	0	2

Relative initial rates are expressed as percentage of the blank reaction rate.
[a] For details, see Section 2.

tetrahydrofuran and *N*-methylpyrrolidone, were not. A slightly activating effect of methanol was observed. Moreover, in some cases α-LA showed to have a protective effect (e.g., when using ethanol and dimethylsulfoxide), whereas different cosolvents, e.g., acetone and dioxane, showed a more significant effect on the LS complex rather than on β-1,4-GalT activity. These data will permit the choice of the best cosolvent to be employed in galactosylation reactions accordingly to the biocatalyst to be used, β-1,4-GalT or the LS complex.

4. Conclusions

In conclusion, it has been shown that, besides its well-known role in lactose synthesis, α-LA can significantly influence the reaction rate of glycosylation of non-natural substrates, like the complex glucosides **1** and **2**. Surprisingly, using these compounds as acceptors, not only α-LA was not necessary, but it showed a significant inhibitory effect. The optimization of the reaction conditions allowed the biocatalyzed preparation and the characterization of two differently glycosylated derivatives of **2**, using UDP-Gal and UDP-Glc as sugar donors. Moreover, the covalent immobilization of β-1,4-GalT on Eupergit C 250 L allowed to evaluate the enzyme activity in the absence of α-LA and to study the synthetic performances of the immobilized biocatalyst. Finally, the best organic cosolvents to be used in galactosylation reactions accordingly to the correspondent biocatalyst, β-1,4-GalT or the LS complex, were identified.

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