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β-Galactosidase transferase activity in ice and use of vinyl-β-D-galactoside as donor

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Abstract: The ability of vinyl-β-D-galactoside as a donor in transglycosylation reactions catalysed by the β-glycosidase from *Aspergillus oryzae* using allylic alcohol and methyl-α-galactoside as acceptors is tested. A kinetic study made in comparison with another donor, the 2-nitrophenyl-β-D-galactoside, shows that the use of the latter leads at room temperature to better yields than those obtained from the former. A reverse situation is observed in ice at −7°C, conditions in which the yield for transglycosylation can be enhanced from 59% to 82% with methyl-α-galactoside as an acceptor. © 1997 Elsevier Science Ltd

Recently, the importance of the biological function of the oligosaccharides has been recognized. The key role played by carbohydrates in biological mechanisms has led to a rapid development of the sugar chemistry. While the regioselective synthesis of the glycoside bond may be achieved by means of the standard chemical approach[1] which usually requires cumbersome and sophisticated protection–deprotection multisteps, the enzymatic synthesis of the saccharides has become, over the last ten years, a very powerful alternative[2]. Two kinds of enzyme are able to catalyse the formation of the glycoside bond: the glycosyltransferases and the glycosylhydrolases. The former generally lead to high yields and total regio- and stereoselectivities but they need very expensive sugar nucleotides as activated donors[3–5]. Furthermore, their high cost and their low stability have greatly limited their use. The latter can also catalyse the formation of the glycosidic linkage not only via the reverse reaction but also via their transferase activity. Due to their high stability and low cost, these enzymes are very attractive since they also induce high selectivities[6–9]. Their main disadvantage comes from the low yields usually obtained since the transferase activity remains in competition with the hydrolysis of the substrate and of the glycoside synthesized. The best results are usually obtained by using a donor bearing a good leaving group at the anomeric position. Thus p- and o-nitrophenylglycosides are the most widely employed for this purpose leading to the synthesis of blood determinant di- and trisaccharides[10–15]. The aim of this paper is to evaluate the potential of vinylglycosides in connection with low temperature enzymatic reactions to enhance the yields of transglycosylated products. The choice of the donor and the temperature study were motivated by comparison with the behaviour of other hydrolytic enzymes. For instance, the use of vinylglycosides avoids the reverse transglycosylation reaction as well as vinyl acetate does in the transesterification catalysed by lipases.

Obviously, the situation is less favourable in the case of glycosidases since they lose their activity in non aqueous media. Proteases also exhibit the transferase activity and a lot of peptides have been synthesized using this property. As in the case of the glycosidases, the conversion observed for the transpepdidation reaction is often low. It has recently been shown that the yields can be largely enhanced when the reactions are monitored at low temperatures in ice[16,17]. To the best of our knowledge, such an approach has never been tested with glycosidases although an hydrolytic activity of a β-galactosidase from a psychotropic bacterium has been demonstrated in a frozen aqueous solution[18].

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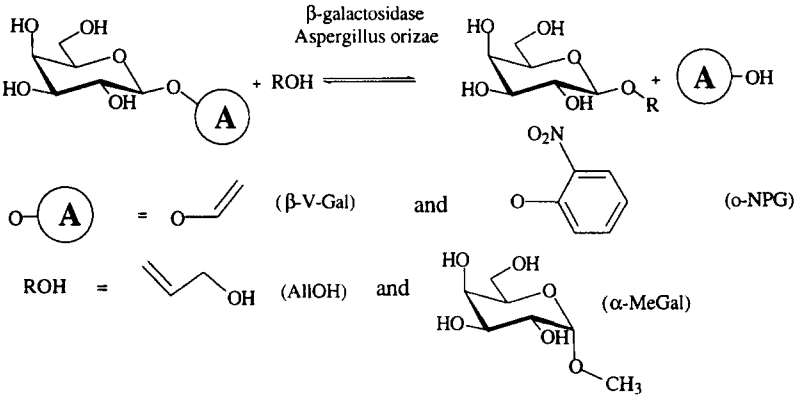
Results and discussion

The model reactions presented in this work are the transglycosylations catalysed by the commercial β-galactosidase from *Aspergillus oryzae*, using vinyl-β-D-galactoside and 2-nitrophenyl-β-D-galactoside as donors, allylic alcohol and methyl-α-D-galactoside as acceptors (see Scheme 1).



Scheme 1. Transglycosylations and reactants used in the study.

The kinetic studies of the reactions were made by means of 250 MHz proton NMR spectroscopy. Each point determining the kinetic curves was the result of one experiment in which the reaction was quenched at a given time. In all cases, it was possible to follow the relative concentrations of the substrates and of the products even for the synthesis of the disaccharides. When several disaccharides were formed, they were identified by conventional one and two dimensional NMR techniques at 500 MHz. Some of their individual resonances were also well resolved at 250 MHz, thus allowing the determination of the molar fraction of each compound.

From the analysis of the NMR spectra, the specificity of the enzyme was shown to be strictly β. Furthermore, independent of the nature of the β-galactosyl donor, the methyl-β-D-galactopyranosyl-(1,6)-α-D-galacto-pyranoside: β-D-Gal-(1,6)-α-D-Gal-OMe (see Figure 1, proton NMR spectra) was the main component of the mixture (see Scheme 2). The low percentages of β-D-Gal-(1,2)-α-D-Gal-OMe and of β-D-Gal-(1,3)-α-D-Gal-OMe, present in the mixtures were also determined via the integration of the corresponding proton NMR signals. Such mixtures also contained very small amounts of trisaccharides. They were disregarded in this study.

Tables 1 and 2 show the maximum percentages of transgalactosylation at three temperatures (20°C, +5°C and −7°C) obtained respectively with AllOH and methyl-α-D-galactoside as acceptors. Two typical kinetic curves are also given in Figs 2 and 3.

From Tables 1 and 2, it is clear that the maximum conversion percentages are obtained after a short time (less than 2.5 h) in all the reactions studied at 20°C. At lower temperatures, this time never exceeds three and a half days. It is rather curious to see that the procedures described in the literature for similar reactions indicate much longer incubation times: about one week under similar conditions. However, the determination of the best moment to quench such reactions is highly important as in a short time some undesirable effects can occur. The transglycosylation of β-V-Gal in the presence of methyl-α-D-galactoside clearly exemplifies this fact (Table 2). After 1.5 h of incubation, a yield of 36% was obtained for the β-D-Gal-(1,6)-α-D-Gal-OMe while 27% of the donor had not reacted. The same yield (35%) was observed after 2.5 h but only 9% of the donor was recovered.

It is obvious that the O-2-nitrophenyl group is a better leaving group than the O-vinyl one. For each reaction, the yields in transglycosylated products are better when using β-o-NPG as a donor. For instance, at 20°C, when the acceptor is AllOH, a total transglycosylation conversion of 45% is observed with β-V-Gal and of 62% with β-o-NPG. A similar situation occurs when the acceptor is

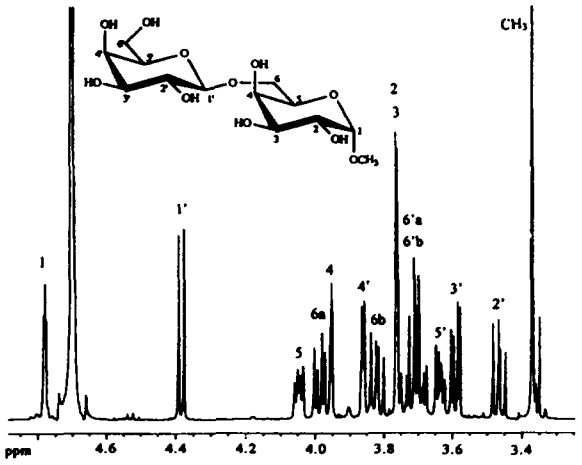
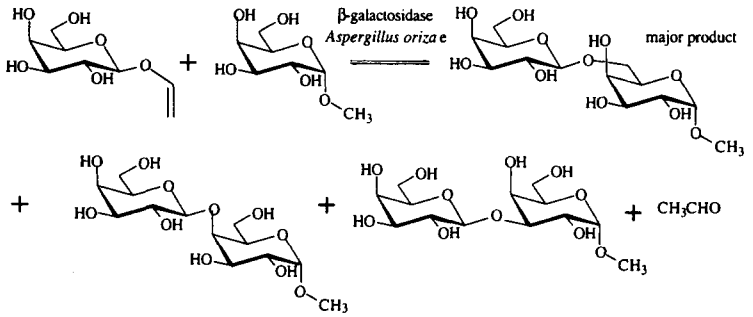


Figure 1. ^1H NMR spectrum (500 MHz) of the β-D-Gal-(1,6)-α-D-Gal-OMe (solvent: D_2O).



Scheme 2. Transglycosylation of vinyl-β-D-galactopyranoside with methyl-α-D-galactopyranoside.

Table 1. Transglycosylation of β-V-Gal and β-o-NPG with allylic alcohol catalysed by β-galactosidase from *Aspergillus oryzae*. Conditions: donor, 1 equivalent; acceptor, 3 equivalents. enzyme, 70 units/mmol of donor

Donor	T (°C)	Time (h)	Relative %		
			Donor	β-allylGal	Galactose
β-V-Gal	20	2,5	15	45	40
β-o-NPG	20	0,75	8	62	30
β-V-Gal	5	6	24	48	28
β-o-NPG	5	3	10	60	30
β-V-Gal	-7	30	12	50	38
β-o-NPG	-7	17	45	30	25

methyl-α-D-galactoside: at 20°C, the sum of the percentages of the disaccharides synthesized with β-V-Gal is 59% and 75% with β-o-NPG. This trend seems to be reversed at −7°C. In fact, this can be understood by considering the relative solubility of the two donors in water. In the crystals of ice, the liquid phase, in which the reactants are present, probably contains smaller amounts of the less soluble β-o-NPG than those of β-V-Gal. Nevertheless, when both of the reactants are sufficiently water soluble, very high conversion is observed. This is particularly true for the transglycosylation of β-V-Gal in the presence of methyl-α-D-galactoside where a total yield of 80% of disaccharides was obtained after about 3 days of incubation (see Table 2 and Figure 3). At this time, all the β-V-

Table 2. Transglycosylation of β-V-Gal and β-o-NPG with methyl-α-D-galactoside catalysed by β-galactosidase from *Aspergillus oryzae*. Conditions: donor, 1 equivalent; acceptor, 3 equivalents. enzyme, 70 units/mmmol of donor

Donor	T (°C)	Time (h)	Relative %				
			Donor	Disaccharides			Galactose
				1,6	1,2	1,3	
β-V-Gal	20	1,5	27	36	12	5	20
β-V-Gal	20	2,5	9	35	18	6	32
β-o-NPG	20	0,75	8	57	12	6	17
β-V-Gal	5	6	29	35	6	2	28
β-o-NPG	5	3	29	46	8	2	15
β-V-Gal	-7	78	0	70	8	2	20
β-o-NPG	-7	78	10	50	5	<1	35

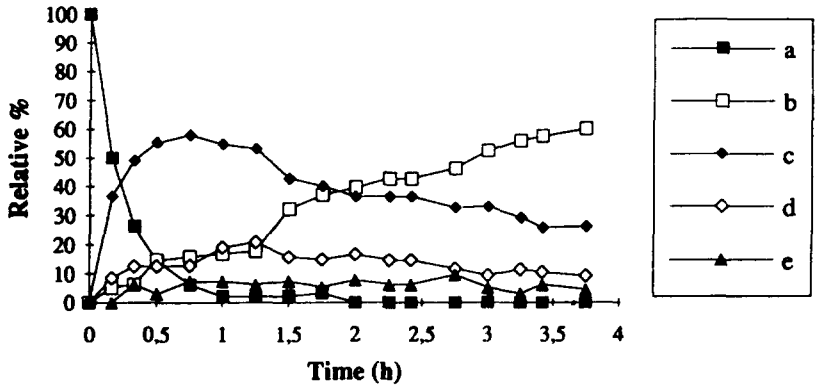


Figure 2. Kinetic study of the transglycosylation reaction of 2-nitrophenyl-β-D-galactopyranoside with methyl-α-D-galactopyranoside as an acceptor. Catalyst: β-galactosidase from *Aspergillus oryzae*. (a=o-NPG, b=galactose, c=1,6-disaccharide, d=1,2-disaccharide, e=1,3-disaccharide).

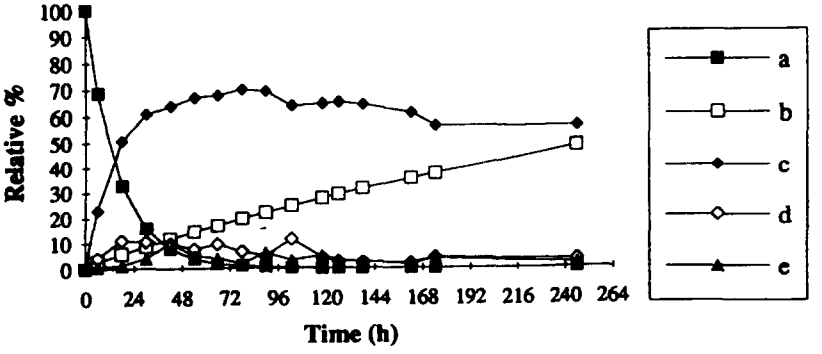


Figure 3. Kinetic study of the transglycosylation reaction of vinyl-β-D-galactopyranoside with methyl-α-D-galactopyranoside as an acceptor. Catalyst: β-galactosidase from *Aspergillus oryzae*. (a=β-V-Gal, b=galactose, c=1,6-disaccharide, d=1,2-disaccharide, e=1,3-disaccharide).

Gal was consumed and only 20% of galactose was formed. This clearly indicates that the hydrolytic activity is considerably reduced in ice. Obviously, the rate of the transglycosylation is also reduced but to a lesser extent. The glycosidases seem to exhibit a similar behaviour to the proteases in ice. The relatively poor results obtained in the reactions with AllOH can also be explained by considering the low solubility of this acceptor in water at −7°C. At a temperature of 5°C, no real gain is observed as the rates of tranglycosylation and of hydrolysis seems to be equally reduced thus showing that the presence of ice is necessary to decrease the hydrolytic activity. Up to now, we have avoided water-organic solvents (such as aqueous DMF for instance) which are known to deactivate partially the enzymatic activity, thus increasing the time course of incubation. Work is in progress in the laboratory to combine organic solvents and suitable donors in order to improve as much as possible the yields of the transglycosylation and the regioselectivity to synthesize disaccharides.

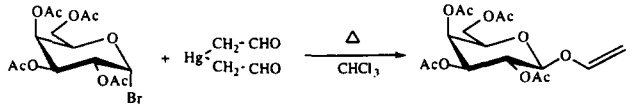
Experimental

Materials and methods

The *Aspergillus oryzae* β-galactosidase (EC 3.2.1.23) was purchased from Sigma (lyophilized powder G7138) and the chemicals supplied by Aldrich were used without further purification. The course of the reactions was followed by means of TLC (precoated silica gel 60 sheets Merk F254). The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker WM250 and a Bruker AX500 spectrometers. The components of the reaction mixtures were separated on charcoal (Darco G-60, 100 mesh, Aldrich)–celite (Fluka 535) columns. Complete analysis of the structures and assignment of each resonances was made using standard 2D sequences (COSY HH and HCOOR correlations).

Synthesis of the vinyl-β-D-galactoside

This compound was easily prepared according to the procedure described in reference[19]:



After removal of the acetyl groups (MeONa/MeOH), the vinyl-β-D-galactoside was obtained in good yield.

Vinyl-β-D-galactopyranoside

^{1}H-NMR (D_2O) δ: 3.35–3.56 (m, 5 H); 3.70 (d, J=2.93 Hz, 1 H); 4.09 (dd, J_{8-7}=6.59 Hz, $J_{8-8'}$=2.2 Hz, H-8); 4.38 (dd, $J_{8'-8}$=2.2 Hz, $J_{8'-7}$=13.91 Hz, H-8′); 4.48 (d, J_{1-2}=7.32 Hz, H-1); 6.32 (dd, J_{7-8}=6.59 Hz, $J_{7-8'}$=13.91 Hz, H-7). ^{13}C-NMR (CD_3COCD_3) δ: 61.04; 68.73; 70.53; 72.83; 75.71; 92.69; 101.71; 149.84.

Kinetic study of the reactions

a) Transglycosylations at 20°C and at 5°C

o-Nitro-β-D-galactoside as donor

0.829 mmol (250 mg) of the donor was mixed with 2.41 mmols of the acceptor in 0.05 M acetate buffer (pH=5). Then, 10.4 mg of the enzymatic preparation (58 units, 70 units/mmol of donor) was added. Aliquots of 250 μL were taken from this solution every 15 minutes during 4 hours. For each sample, the reaction was quenched with the addition of 10 μL of 1 M NaOH. Then, the water was removed under reduced pressure and the dry powder thus obtained was redissolved in D_2O. The resulting solution was submitted to proton NMR analysis.

Vinyl-β-D-galactoside as donor

The experiments were conducted similarly, but the samples were taken at t=10 min, 20 min, 35 min, 1 h, 1 h 25 min, 1 h 50 min, 2 h 35 min, 3 h 20 min, 4 h 5 min, 5 h, 6 h, 7 h, 8 h, 9 h and 10 h.

b) Transglycosylations at −7°C

o-Nitro-β-D-galactoside as donor

Allylic alcohol as acceptor. At −7°C, the solid reaction mixture makes the sampling impossible. So, the kinetic study was managed as follows: 16 vials containing the same quantities of compounds were incubated at −7°C. For each of these vials, the reaction was quenched at different times (6, 17, 24, 41, 54, 65, 78, 88.5, 101, 161, 189.5, 213.5, 233, 257, 329 and 360 hours) by means of the addition of 10 μL of 1 M NaOH. Then, the water was removed under reduced pressure and the dry powder thus obtained was redissolved into D_2O. The resulting solution was submitted to proton NMR analysis.

Each vial was composed of 12 mg of the donor (39.8 μmols), 125 μL of 0.05 M acetate buffer (pH=5), 7.8 μL of allylic alcohol (114.3 μmols). This solution was homogenised with a vortex and 50 μL of an enzyme solution (10 mg/mL of the lyophilized powder, 2.8 units) was added. The resulting mixture was rapidly homogenised with a vortex, cooled at −15°C for 5 minutes then the vial was transferred to a regulated cryostat at −7°C.

Methyl-α-D-galactoside as acceptor. A similar procedure was used apart from the beginning of the preparation of the 12 samples: 12 mg of the donor (39.8 μmols) was introduced into a flask containing a solution of 22 mg (114.3 μmols) of the acceptor in 125 μL of the buffer. 2.8 Units of the enzyme in 50 μL of the buffer was added to this solution.

Vinyl-β-D-galactoside as donor

Allylic alcohol as acceptor. similar procedure as above apart the quantities of the components in each vial. Into each of them was introduced 10 mg (48.5 μmols) of the donor in 50 μL of buffer, 150 μL of the buffer and 9.5 μL of the acceptor (139.3 μmols). The mixture was homogenised with a vortex.Then, 3.4 units (0.6 mg of the lyophilized powder in 70 μL of buffer) of the enzyme was added and the solution was homogenised with a vortex. Then a similar procedure to that above was applied. The reactions were quenched at t=6, 18, 30, 42, 54, 66, 78, 90, 103, 118, 126, 138, 162, 174, 246 hours.

Methyl-α-D-galactoside as acceptor. Similar procedure and quantities as those above.

Synthesis of the β-D-Gal-(1,6)-α-D-Gal-OMe at −7°C

1 mmol of β-V-Gal (206 mg) and 3 mmols of the methyl-α-D-galactoside (582 mg) were dissolved in 1.7 mL of the 0.05 M acetate buffer (pH=5) and cooled at 0°C. In another flask, 70 units (12 mg) of the lyophilized preparation of the β-galactosidase (*Aspergillus oryzae*, Sigma G7138) was dissolved in 1 mL of buffer and cooled at 0°C. The enzyme solution was introduced into the reactor with the substrates and immediately cooled at −15°C for 5 minutes. Then, the mixture was transferred to a cryostat regulated at −7°C. The reaction was allowed to proceed for 78 hours. At this time, the reaction was quenched by means of the addition of 250 μL of NaOH 1 M.

The components of the reaction mixture were separated on a charcoal–celite (1/1, w/w) column with a gradient of ethanol/water. The eluent is sucked through the column by means of a Gilson Minipuls 3 pump. The flow of the collector was 400 drops/tube (7.5 mL, 12 minutes). The tubes 1–15, 16–35 were respectively eluted with a 5% and with a 10% ethanol/water mixtures. The contents of each tube were examined by TLC (see above) using the Seymour eluent ($CHCl_3$/MeOH/AcOH/H_2O 60:30:3:5). The fractions 4–5 contained the mixture of α- and β-galactose (33 mg, R_f=0.47). The fractions 6–14 yielded the unreacted methyl-α-D-galactoside (405 mg, R_f=0.64) while the tubes 20–23 contained a mixture of the three 1,6-, 1,4- and 1,3-disaccharides (165 mg, largely 1,6, respective R_f=0.28, 0.36, 0.20). The last 24–29 fractions contained the nearly pure β-D-Gal-(1,6)-α-D-Gal-OMe (102 mg). The total yield calculated for the 1,6-disaccharide was 67%.

Methyl-β-galactopyranosyl-1,6-α-galactopyranoside

^{1}H-NMR (D_2O) δ: 3.37 (s, CH_3); 3.47 (dd, $J_{2'-1'}$=7.74 Hz, J_{2-3}=9.90 Hz, H-2′); 3.57 (dd, $J_{3'-2'}$=9.81 Hz, $J_{3'-4'}$=3.38 Hz, H-3′); 3.63 (ddd, $J_{5'-6'a}$=4.45 Hz, $J_{5'-6'b}$=7.85 Hz, $J_{5'-4'}$=1 Hz, H-5′); 3.69 (dd, $J_{6'a-5'}$=4.30 Hz, $J_{6'a-6'b}$=11.50 Hz, H-6′a); 3.73 (dd, $J_{6'b-5'}$=7.75 Hz, $J_{6'b-6'a}$=11.50 Hz, H-6′b); 3.76 (m, H-2 and H-3); 3.82 (dd, J_{6b-6a}=11.25 Hz, J_{6b-5}=7.65 Hz, H-6b); 3.86 (dd, $J_{4'-3'}$=3.6 Hz, $J_{4'-5'}$=0.9 Hz, H-4′); 3.95 (d, J=1.5 Hz, H-4); 3.99 (dd, J_{6a-6b}=11.03 Hz, J_{6a-5}=4.50 Hz, H-6a); 4.05 (ddd, J_{5-6b}=7.50 Hz, J_{5-6a}=4.50 Hz, J_{5-4}=0.9 Hz, H-5); 4.38 (d, H-1′); 4.78 (d, H-1). ^{13}C-NMR (D_2O) δ: 52.0 (CH_3); 57.6 (C-6′); 64.7 (C-2); 65.2 (C-4′); 65.7 (C-4); 65.8 (C-6); 65.9 (C-3); 66.1 (C-5); 67.4 (C-2′); 69.3 (C-3′); 71.6 (C-5′); 96.2 (C-1); 99.9 (C-1′).

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