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Bacillus circulans β-galactosidase catalyses the synthesis of N-acetyl-lactosamine in a hydro-organic medium via a steady-state ordered Bi Bi reaction mechanism

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ABSTRACT

Starting from a galactosyl donor, the β-galactosidase from *Bacillus circulans* (Lactoles® L3; Bcβ-Gal) catalyses the transgalactosylation of N-acetyl-D-glucosamine (GlcNAc) in hydro-organic media, leading to the synthesis of N-acetyl-lactosamine (LacNAc). The aim of this study was to determine the kinetic properties of LacNAc synthesis and sought to highlight the effect of the organic solvent on the enzyme kinetic parameters. Analyses of enzyme kinetics were performed in aqueous solutions and water/*tert*-butanol mixtures. A steady-state ordered Bi Bi mechanism was characterised for the transgalactosylation of GlcNAc catalysed by Bcβ-Gal in water/*tert*-butanol (70:30, v/v) mixtures, starting from *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as a galactosyl donor. Moreover, *tert*-butanol was shown to have various effects on the kinetic parameters of the total transformation of *o*NPG and lactose catalysed by Bcβ-Gal, which led us to liken this solvent to a noncompetitive inhibitor towards the former and a competitive inhibitor towards the latter.

1. Introduction

N-acetyl-lactosamine (LacNAc) βGal-(1-4)-GlcNAc is one of the most representative core structures in oligosaccharide components of glycoproteins and glycolipids, both of which play a central role in many cellular recognition phenomena [1] and can be involved in various diseases such as infections [2], autoimmune and inflammatory pathologies [3–5] or cancer [6]. This disaccharide can be produced by an enzymatic transgalactosylation reaction catalysed by the β-galactosidase from *Bacillus circulans* (Lactoles® L3; Bcβ-Gal; EC 3.2.1.23, glycoside hydrolase family 42). During the reaction, which is carried out in aqueous or hydro-organic media [7–13], a galactose moiety is transferred from a galactosyl donor, such as *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose, to N-acetyl-D-glucosamine (GlcNAc). Three parallel reactions can occur during this process (Scheme 1), depending on the nature of the nucleophile attacking the galactosyl-enzyme intermediate, once it is formed. Like all glycosidases, Bcβ-Gal is indeed known not only to catalyse the transfer reaction producing a galactosylated derivative of GlcNAc such as LacNAc (βGal-(1-4)-GlcNAc) and N-acetyl-allolactosamine (βGal-(1-6)-GlcNAc) (C) [14] but also to catalyse the autocondensation (B) and the hydrolysis (A) of the galactosyl donor. Bcβ-Gal is a retaining β-galactosidase that is assumed to operate through a double displacement mechanism, both steps involving a transition state with a substantial oxocarbenium ion character [15–18]. According to studies on the kinetics of the hydrolysis or transglycosylation activity of various glycosidases, a glycosidase-catalysed reaction involves either a ping pong Bi Bi [19–23] or an ordered (Uni Bi or Bi Bi) kinetic mechanism [10,24–26]. The ping pong and ordered Bi Bi mechanisms are both special multisubstrate reactions. However, for a two-substrate, two-product system, the ping pong Bi Bi mechanism involves an enzyme E reacting with one substrate A to form a product P and a modified enzyme E′, the latter then reacting with a second substrate B to form a second, final product Q, and regenerating the original enzyme E. The ordered mechanism, on the contrary is characterised by the fact that one substrate A has to bind to the enzyme E first, followed by the other substrate B, to form a ternary-complex E·A·B and to allow catalysis to occur: once formed, products P and Q are released from the enzyme E in a distinct order.

Very recently, we have shown that the synthesis of LacNAc can be carried out efficiently and regioselectively in aqueous media highly concentrated in substrates and in water/*tert*-butanol (*t*-BuOH) (70:30, v/v) mixtures, using Bcβ-Gal [14]. The present paper reports on the use of initial velocity measurements to characterise the kinetics of the total transformation of the galactosyl donors *o*NPG and lactose that occurs during the transgalactosylation of GlcNAc. The parameters of the reaction kinetics were determined

Abbreviations: Bcβ-Gal, β-galactosidase from *Bacillus circulans*; E, enzyme; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; LacNAc, N-acetyl-lactosamine; NP-HPLC, normal-phase high pressure liquid chromatography; *o*NP, *o*-nitrophenol; *o*NPG, *o*-nitrophenyl-β-D-galactopyranoside; *t*-BuOH, *tert*-butanol.

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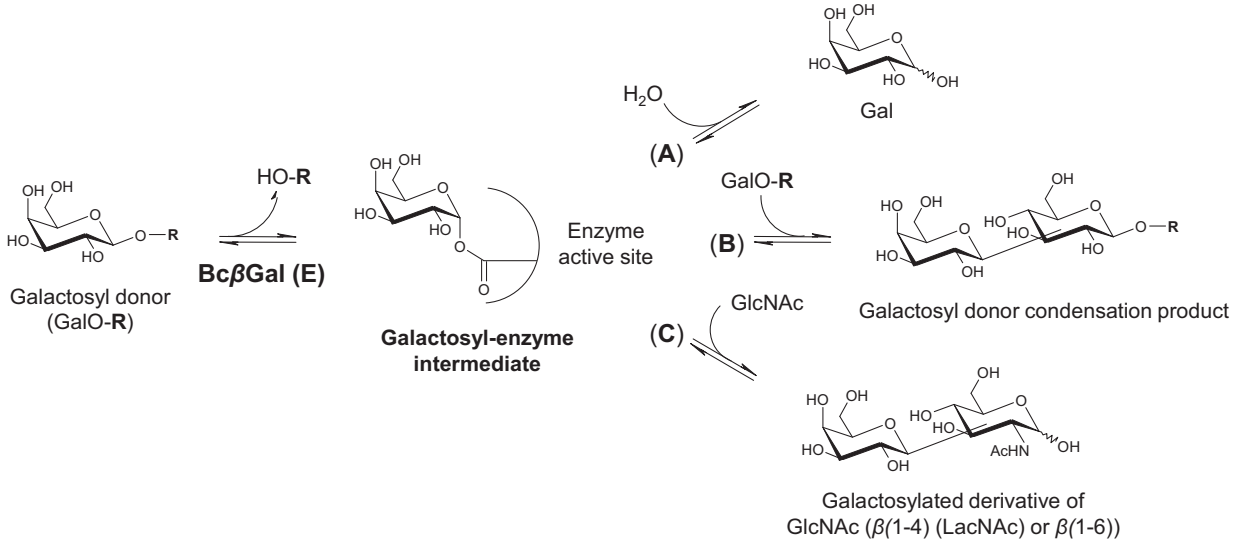


Scheme 1. Parallel reactions occurring during the GlcNAc transgalactosylation catalysed by BcβGal, starting from *o*NPG or lactose as a galactosyl donor. Galactosyl donor condensation products and galactosylated derivatives of GlcNAc can also be further substrates for secondary hydrolysis reactions.

in water and water/*t*-BuOH (70:30, v/v) mixtures. The effects of *t*-BuOH on these reaction kinetics will be discussed. Moreover, the kinetic mechanism of the transgalactosylation of GlcNAc catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures using *o*NPG as a galactosyl donor was also demonstrated.

2. Materials and methods

2.1. Enzyme and chemicals

All chemicals were purchased from Sigma Co. (USA). Deionised water was obtained using a Milli-Q system (Millipore, France).

Lactoles® L3 is a soluble commercial preparation of β-galactosidase from *B. circulans* (EC 3.2.1.23, glycoside hydrolase family 42; BcβGal), manufactured by Daïwa Kasei K.K. (Japan), with an activity of 37,851 U mL^{-1}. One unit is defined as the amount of enzyme which releases 1 μmol *o*-nitrophenol per minute under the standard conditions described below. This enzyme preparation was withdrawn some years ago by Daïwa Kasei K.K. and is no longer available.

2.2. Measurement of the BcβGal activity

Initial velocity measurements were performed at 40 °C with a solution of the crude preparation of BcβGal diluted in 100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$) (1:250 dilution; 151 U mL^{-1}), on a chromogenic substrate (*o*NPG). Continuous changes in the absorbance at 415 nm were monitored in spectrophotometric cuvettes of 1 cm path length using a UV–visible spectrophotometer (Lambda 650; Perkin Elmer), associated with a PTP1 (Peltier temperature programmer) system. Reaction mixtures (2 mL) containing the substrate (concentration range from 1 to 20 mM) and 100 mM sodium phosphate buffer, pH 6.5 (10 mM $MgCl_2$), were incubated in the spectrophotometer for 10 min prior to addition of 20 μL of the enzyme solution, under stirring conditions. The initial release velocity of *o*-nitrophenol was then measured in OD min^{-1} at 415 nm from the linear relationship of the *o*-nitrophenol concentration with reaction time (0–5 min) using UV Winlab 5 software. The molar extinction coefficient of *o*-nitrophenol (150 L mol^{-1} cm^{-1}) was determined under the experimental conditions using a calibration curve established from *o*-nitrophenol concentrations.

2.3. Enzyme assays of steady-state kinetics

For all enzyme assays, except if otherwise stated in the text, the aqueous phase was not buffered given that the ultrapure water used had a pH close to 6, which is optimal for BcβGal activity [10]. Potential issues due to the presence of salts and their effects, notably in aqueous organic solvent mixtures, were thus avoided.

2.3.1. Kinetic characterisation of the total transformation of oNPG

Initial velocity measurements were performed at 40 °C using the procedure described above (see Section 2.2). The reaction mixtures (3 mL) containing various amounts of substrates (5–50 mM *o*NPG and 0–50 mM GlcNAc) solubilised in water/*t*-BuOH (70:30, v/v) mixtures, in 100 mM sodium phosphate buffer, pH 6.5 (10 mM $MgCl_2$), or in water, were incubated in the spectrophotometer for 10 min prior to addition of the appropriate volume of the crude preparation of BcβGal, or of a diluted solution of this crude preparation previously prepared in the reaction solvent, giving the catalytic activity concentrations stated in the text, within the range 1.5–30 U mL^{-1}. The initial release velocity of *o*-nitrophenol was then measured. The molar extinction coefficient of *o*-nitrophenol (328 L mol^{-1} cm^{-1} in water and 260 L mol^{-1} cm^{-1} in a water/*t*-BuOH (70:30, v/v) mixture) was determined under the experimental conditions using a calibration curve established from *o*-nitrophenol concentrations.

2.3.2. Kinetic characterisations of the total transformation of lactose and of the GlcNAc transgalactosylation

Initial velocity measurements were performed at 40 °C in water/*t*-BuOH (70:30, v/v) mixtures or in water, under stirring conditions. Reaction mixtures (3 mL) containing various amounts of substrate (5–50 mM) were incubated for 10 min prior to addition of the appropriate volume of the crude preparation of BcβGal, or of a diluted solution of this crude preparation previously prepared in the reaction solvent, giving the catalytic activity concentrations stated in the text, within the range 9–37.5 U mL^{-1}. Samples (100 μL) were taken at regular intervals and treated at 100 °C for 5 min to inactivate the enzyme. The decrease in lactose concentration or the increase in concentration of galactosylated derivatives of GlcNAc was monitored over time by NP-HPLC. To rule out potential chemical hydrolysis of lactose and galactosylated derivatives of GlcNAc due to the 5 min thermal treatment, samples containing

5 or 50 mM of lactose or LacNAc were treated at 100 °C for 5 min in water/*t*-BuOH (70:30, v/v) mixtures or in water, under stirring conditions. They were then cooled down for 5 min in an ice bath and analysed by NP-HPLC, showing that neither lactose nor LacNAc underwent chemical hydrolysis in these experimental conditions. The initial velocities were calculated from the linear relationship of the lactose concentration or of the total concentration of galactosylated derivatives of GlcNAc against reaction time (0–10 min).

For the product inhibition study, initial velocity measurements of the GlcNAc transgalactosylation were performed using the same procedure, except that the reaction mixtures (1.5 mL) were composed of various amounts of *o*NPG (5–50 mM), GlcNAc (50 mM) and *o*NP (0–5 mM).

2.4. Data analysis

Except for the product inhibition study, all initial rate data were fitted to the equation that describes the steady-state sequential ordered Bi Bi mechanism equation (Eq. (1)) using nonlinear least-squares regression analysis in the Enzyme Kinetics Module of Sigma Plot, "Enzyme Kinetics 2004 1.2" (Systat Software Inc., San Jose, USA).

$$v = \frac{V_{max}[A][B]}{K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]} \quad (1)$$

Initial rate data corresponding to the product inhibition study were fitted to all the inhibition models in the "Enzyme Kinetics 2004 1.2" software using nonlinear least-squares regression analysis. The equation for full noncompetitive inhibition (Eq. (2)) was relevant to the work presented here.

$$v = \frac{V_{max}[A]}{(K_{mA} + [A])(1 + [I]/K_i)} \quad (2)$$

A and B represent the substrates *o*NPG and GlcNAc, respectively. K_{mA} and K_{mB} are the Michaelis constants for A and B, respectively. V_{max} is the maximum velocity. K_{ia} is defined as the dissociation constant of A from the binary complex E·A and K_i is the inhibition constant for the inhibitor used [27].

To visualise the quality of fitting, the experimental data points are presented in plots along with theoretical lines fitted by "Enzyme Kinetics 2004 1.2" software.

2.5. HPLC analyses

Structural and quantitative analyses of lactose or galactosylated derivatives of GlcNAc were conducted using a LC/MS-ES system from Agilent (1100 LC/MSD Trap mass spectrometer VL), with an Uptisphere 6 DIOL normal-phase column (250 mm × 4 mm, 5 μm) eluted with acetonitrile/water/acetic acid (80:20:0.1, v/v/v) at room temperature and at a flow rate of 1 mL min^{-1}. Lactose was detected and quantified by differential refractometry while galactosylated derivatives of GlcNAc were detected and quantified by ultraviolet absorption at 210 nm using a previously established procedure [14], and HP Chemstation software off-line for the processing.

2.6. Solubility analyses

The solubility of *o*NPG, lactose and GlcNAc in water or water/*t*-BuOH (70:30, v/v) mixtures was evaluated at 40 °C under stirring conditions by progressively adding into a sample containing the compound (100 mg of *o*NPG or 500 mg of lactose or GlcNAc), various volumes of solvent decreasing within the range 10–1000 μL, the starting volume depending on the compound tested, until complete solubilisation was obtained. The molar concentration of the compound after its complete solubilisation was then considered as its maximal solubility.

3. Results and discussion

3.1. Effect of t-BuOH on the kinetic characterisation of the total transformation of oNPG and lactose catalysed by Bcβ-Gal in aqueous solutions and water/t-BuOH (70:30, v/v) mixtures

The total transformation of *o*NPG, which occurs during the transgalactosylation of GlcNAc catalysed by Bcβ-Gal, was monitored in initial velocity experiments conducted in water/*t*-BuOH (70:30, v/v) mixtures using various GlcNAc concentrations within a range 5–50 mM in order to evaluate the kinetic mechanism of the enzyme. The reaction was also carried out in the absence of GlcNAc in water, in 100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$), or in water/*t*-BuOH (70:30, v/v) mixtures; the aim in every case was to identify the kinetic constants for Bcβ-Gal in water and water/*t*-BuOH (70:30, v/v) mixtures. The total transformation of lactose catalysed by Bcβ-Gal was also studied in water and water/*t*-BuOH (70:30, v/v) mixtures. The transformation of *o*NPG and lactose was monitored through the release of *o*-nitrophenol and the disappearance of lactose in the media, respectively.

Systematic analysis of the rates, which were dependent on the substrate concentration, revealed two Lineweaver–Burk double reciprocal plots, depending on whether the galactosyl donor or GlcNAc was parametric. The results concerning the experiments performed with *o*NPG as a galactosyl donor are shown in Fig. 1. Kinetic constants determined for all experiments are presented in Table 1. These results provide information about the effects of *t*-BuOH on the catalytic activity of Bcβ-Gal.

(1) First, it was found that the apparent initial velocity ($V_{max,app}$) of the total transformation of *o*NPG was about 2.5-fold higher in water ($V_{max,app}$ = 8967 μmol min^{-1} mL^{-1}; entry 6) than in a water/*t*-BuOH (70:30, v/v) mixture ($V_{max,app}$ = 3478 μmol min^{-1} mL^{-1}; entry 1). Thus *t*-BuOH seemed to negatively affect the catalytic activity of Bcβ-Gal.

(2) Secondly, the $K_{m,app}$ of *o*NPG was found to increase from 9.0 mM in water (entry 6) to 14.8 mM in a water/*t*-BuOH (70:30, v/v) mixture (entry 1), which characterised a lack of affinity of Bcβ-Gal for *o*NPG in the presence of *t*-BuOH, most probably due to the solvation of the enzyme by *t*-BuOH. This solvent might indeed enter the catalytic site and sterically hinder *o*NPG. Logically, the decrease in the $V_{max,app}$ associated with the increase in the $K_{m,app}$ of *o*NPG observed when using *t*-BuOH resulted in a higher catalytic efficiency in water than in a water/*t*-BuOH (70:30, v/v) mixture.

(3) Thirdly, the effects of *t*-BuOH on the kinetic parameters of the lactose transformation catalysed by Bcβ-Gal were shown to be slightly different. While the $V_{max,app}$ values in water (entry 9) and in a water/*t*-BuOH (70:30, v/v) mixture (entry 8) were similar, demonstrating that the catalytic activity of lactose transformation was not affected by the use of *t*-BuOH, the $K_{m,app}$ of lactose was found to be 36-fold higher in a water/*t*-BuOH (70:30, v/v) mixture than in water. These results are in accordance with those provided by the work of Hancock et al. [28], which showed that the addition of acetonitrile as an organic co-solvent led to a dramatic loss of activity when the thermophilic β-glycosidase from *Sulfolobus solfataricus* was used to transform *p*-nitrophenyl-β-D-galactopyranoside, due to the increase of K_m in conjunction with the higher concentration of acetonitrile. Similar results were described by Takahashi et al. [29] who studied the *Rhizopus niveus* glucoamylase-catalysed hydrolysis of maltose in the absence or presence of

Table 1
Summary of apparent kinetic constants for the total transformation of *o*NPG and lactose catalysed by BcβGal in aqueous media or water/*t*-BuOH (70:30, v/v) mixtures.

Entry	Experimental conditions				$V_{max,app}$ (μmol min^{-1} mL^{-1})[a]	$K_{m,app}$ of galactosyl donor (mM)	Catalytic efficiency ($V_{max,app}/K_{m,app}$) (min^{-1} mL^{-1})
	Galactosyl donor (5–50 mM)	Solvent	[GlcNAc] (mM)	[BcβGal] (U mL^{-1})			
1	*o*NPG	Water/*t*-BuOH (70:30, v/v)	0	30	3478 ± 240	14.8 ± 2.5	0.24
2			10	30	3588 ± 263	12.6 ± 2.5	0.28
3			20	30	3731 ± 93	9.8 ± 0.7	0.38
4			35	30	3796 ± 250	9.4 ± 1.8	0.40
5			50	30	3824 ± 120	8.0 ± 0.8	0.48
6		Water	–	1.5	8967 ± 71	9.0 ± 0.2	1.00
7		100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$)	–	1.5	37,851 ± 1318	3.7 ± 0.5	10.2
8	Lactose	Water/*t*-BuOH (70:30, v/v)	–	9	3741 ± 880	166.8 ± 45	0.02
9		Water	–	37.5	2856 ± 101	4.6 ± 0.7	0.62

[a] Initial velocities were calculated in micromoles of substrate transformed per minute and per millilitre of non diluted enzyme solution.

Table 2
Log *P* values and solubility at 40 °C in water or water/*t*-BuOH (70:30, v/v) mixtures of *o*NPG, lactose and GlcNAc.

Substrate	Log *P*[a]	Substrate's maximal solubility at 40 °C (mM)[b]	
		In water	In water/*t*-BuOH (70:30, v/v)
*o*NPG	−0.78	77 ± 1	117 ± 4
Lactose	−3.41	1126 ± 10	500 ± 20
GlcNAc	−2.48	1930 ± 78	888 ± 28

[a] Log *P* values obtained from the database ACD/log *P* DB.
[b] Averaged triplicate experimental data obtained according to a procedure described in Section 2.6.

acetonitrile. The explanation given for the same phenomenon observed with *o*NPG may also apply here since the preferential protein–solvent binding is most likely responsible for this drastic fall in enzyme affinity for lactose, which is amplified with this substrate because of its high hydrophilicity. Lactose is indeed much more hydrophilic than *o*NPG and is consequently less soluble in a water/*t*-BuOH (70:30, v/v) mixture, as can be seen by comparing their log *P* values and their maximal solubilities in water and in a water/*t*-BuOH (70:30, v/v) mixture (Table 2). Besides, the decreased solubility of lactose in a water/*t*-BuOH (70:30, v/v) mixture could limit its diffusion into this medium, and thus its availability for the enzyme.

Based on these observations, *t*-BuOH seemed to almost act as a noncompetitive inhibitor towards *o*NPG, the $V_{max,app}$ of the total transformation of *o*NPG decreasing in the presence of *t*-BuOH while the $K_{m,app}$ of *o*NPG increased but remained in the same order of magnitude. In the case of the total transformation of lactose catalysed by BcβGal, *t*-BuOH could rather be likened to a competitive inhibitor acting by steric hindrance. A first hypothesis could be that the effects observed here may be attributed to a denaturing effect on BcβGal induced by the use of *t*-BuOH, especially under high concentration conditions (30%, v/v). The partial denaturation of the enzyme could lead to a decrease in the $V_{max,app}$ of the total transformation of *o*NPG. However, the denaturing effect would not explain the significant increase observed for the $K_{m,app}$ of lactose, particularly as it is not predominant under our experimental conditions. Indeed, we have previously shown that BcβGal loses only 10% of its catalytic activity after a 10 min incubation at 40 °C in a water/*t*-BuOH (70:30, v/v) mixture, compared with pure water, with a half-life of about 2.6 h [14]. Another hypothesis could be that the inhibitor effect of *t*-BuOH is related to a differential solvation of the galactosyl donor in water and in water/*t*-BuOH (70:30, v/v) mixtures but this possibility is remote given that the increase in the $K_{m,app}$ observed for both galactosyl donors was much greater for lactose although it had a lower solubility in a water/*t*-BuOH (70:30, v/v) mixture than in water. According to Bell et al. [30], it is generally admitted that a substrate presenting a strong affinity for the solvent, hence highly solvated, would show a decreased affinity for the biocatalyst. However, these authors insisted on the necessity of also taking into account the effect of the potential presence of the solvent in the enzyme's active site. This effect is most likely predominant in our case.

(4) Furthermore, a fourth effect of the presence of *t*-BuOH was found regarding the apparent initial velocities of the total transformations of *o*NPG and lactose in water. The catalytic activity of BcβGal was about 3-fold higher for the transformation of *o*NPG ($V_{max,app}$ = 8967 μmol min^{-1} mL^{-1}; entry 6) than for the transformation of lactose ($V_{max,app}$ = 2856 μmol min^{-1} mL^{-1}; entry 9), meaning that the velocity of formation of the galactosyl-enzyme intermediate was favoured when using *o*NPG as a galactosyl donor. This was most likely due to the fact that *o*-nitrophenol is a better leaving group than D-glucose [31]. In water/*t*-BuOH (70:30, v/v) mixtures, this difference was not found: the $V_{max,app}$ of transformation of *o*NPG and lactose were very similar in these media, close to 3600 μmol min^{-1} mL^{-1} (entries 1 and 8). This phenomenon may be explained by the presence of *t*-BuOH in the enzyme's active site, which might affect the formation of the galactosyl-enzyme intermediate and thus the displacement of the leaving group, namely *o*-nitrophenol, by preventing either the transfer of protons occurring during the double displacement mechanism or the stabilisation of the oxocarbenium ion transition states [17,18].

(5) Apart from the effects of *t*-BuOH on the catalytic activity of BcβGal, the enzyme was also shown to be most active in 100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$). Under these conditions (entry 7), the $V_{max,app}$ of the total

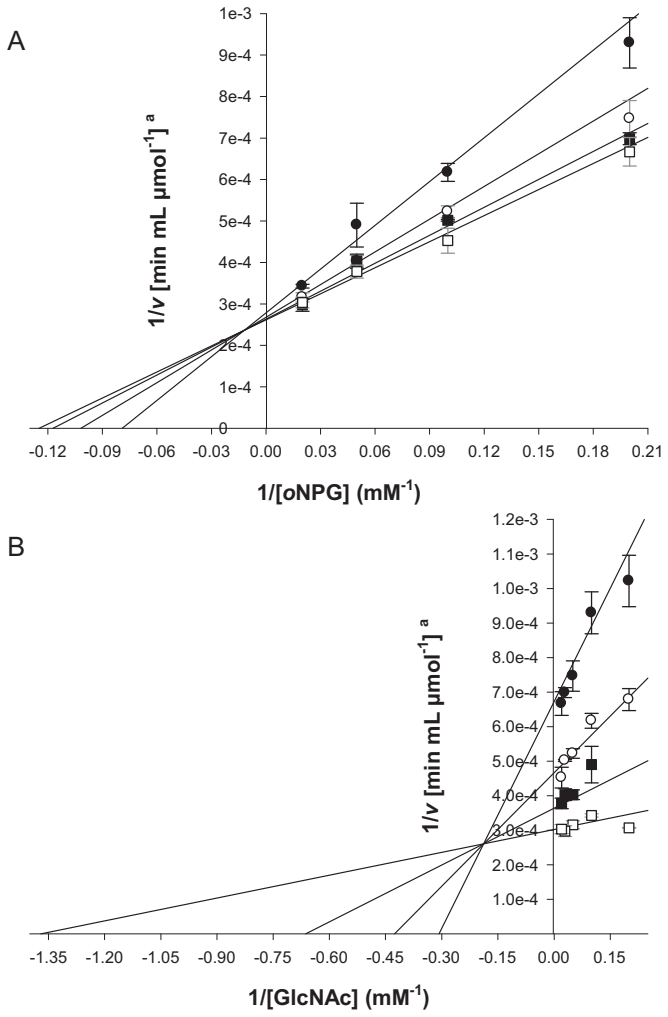


Fig. 1. Steady-state kinetics analysis of the total transformation of *o*NPG that occurs during the GlcNAc transgalactosylation reaction catalysed by BcβGal (30 U mL^{-1}). Double reciprocal plots of $1/v$ versus 1/[*o*NPG] with varying *o*NPG concentrations (5–50 mM) are shown (A). The concentration of GlcNAc was fixed at 10 mM (●), 20 mM (○), 35 mM (■), and 50 mM (□). Double reciprocal plots of $1/v$ versus 1/[GlcNAc] with varying GlcNAc concentrations (5–50 mM) are shown (B). The concentration of *o*NPG was fixed at 5 mM (●), 10 mM (○), 20 mM (■) and 50 mM (□). The steady-state apparent parameters obtained from these bi-substrate analyses are given in Table 1. The data represent the averages of triplicate runs. [a]Initial velocities were calculated in micromoles of substrate transformed per minute and per millilitre of non diluted enzyme solution.

transformation of *o*NPG as well as the $K_{m,app}$ of *o*NPG were the most favourable, logically leading to the higher catalytic efficiency of 10.2 min^{-1} mL^{-1}. The use of a buffered solution at pH 6.5 and/or the presence of Mg^{2+} ions, known to favour the activity of β-galactosidases [32–34], enhanced the catalytic activity of BcβGal in aqueous media, as shown by comparing the catalytic efficiencies obtained in water and in 100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$). Besides, the total transformation of *o*NPG occurring in aqueous solutions under conditions of low substrate concentrations corresponded in all likelihood exclusively to its hydrolysis (entries 6 and 7). Water availability was probably not limited in this case and the hydrolysis of *o*NPG was more efficient than its autocondensation.

(6) Finally, when the concentration of GlcNAc was increased in water/*t*-BuOH (70:30, v/v) mixtures, it was found that the $V_{max,app}$ of the total transformation of *o*NPG increased in conjunction with the increase in GlcNAc concentration, varying from 3478 µmol min^{-1} mL^{-1} without GlcNAc to 3824 µmol min^{-1} mL^{-1} with 50 mM GlcNAc (entries 1–5). At the same time, the $K_{m,app}$ of *o*NPG decreased, indicating that the affinity of the enzyme for *o*NPG increased. Hence, GlcNAc seemed to act both as a substrate and an activator of the GlcNAc transgalactosylation reaction (see path C of Scheme 1). This phenomenon was not due to a potential influence of GlcNAc on the previously described solvation issues related to the presence of *t*-BuOH, which is an inhibitor of the reaction. GlcNAc is indeed hydrophilic (log $P = -2.48$), with a solubility in water/*t*-BuOH (70:30, v/v) mixtures about 2-fold lower than in water (see Table 2). For these reasons, the probability that GlcNAc interacts with *t*-BuOH and thus contributes to limiting the inhibition of BcβGal is very low. Nevertheless, this may be consistent with a sequential (ternary) enzymatic mechanism of the GlcNAc transgalactosylation reaction. This hypothesis was enforced by the fact that the data plotted in the form $1/v$ against both 1/[*o*NPG] and 1/[GlcNAc] gave a series of straight lines intersecting at a point left of the *y*-axis of the double reciprocal plot (Fig. 1), which is consistent with a steady-state sequential ternary-complex kinetic mechanism [27].

In order to confirm this hypothesis, we then focussed on the GlcNAc transgalactosylation reaction by monitoring the kinetics of the production of galactosylated derivatives of GlcNAc (see path C of Scheme 1).

3.2. Kinetic characterisation of the transgalactosylation of GlcNAc catalysed by BcβGal in water/t-BuOH (70:30, v/v) mixtures using oNPG as a galactosyl donor

All experiments were conducted in water/*t*-BuOH (70:30, v/v) mixtures, starting from *o*NPG as a galactosyl donor. The time course of GlcNAc transgalactosylation over 10 min revealed the formation of three galactosylated derivatives of GlcNAc, which had already been structurally identified in a previous work: βGal-(1-4)-GlcNAc (LacNAc), βGal-(1-6)-GlcNAc (N-acetyl-allolactosamine; allo-LacNAc) and a trisaccharide βGal-(1-4)-βGal-(1-4)-GlcNAc that resulted from the transgalactosylation of the LacNAc synthesised [14]. To kinetically characterise the transgalactosylation of GlcNAc catalysed by BcβGal, the sum of the rates of formation of these three compounds was used, giving the rate of GlcNAc transgalactosylation. Nonetheless, LacNAc was shown to be predominantly synthesised in comparison with the two other compounds under all experimental conditions used (data not shown) and the rate of GlcNAc transgalactosylation consisted in fact mainly in the rate of LacNAc formation. For instance, the experimental conditions involving 50 mM of both *o*NPG and GlcNAc gave the high rate of GlcNAc transgalactosylation of 330 µmol min^{-1} mL^{-1}: under these conditions, the rate of LacNAc formation (281 µmol min^{-1} mL^{-1}) was 41-fold and 7-fold higher than those of allo-LacNAc (7 µmol min^{-1} mL^{-1}) and of the trisaccharide βGal-(1-4)-βGal-(1-4)-GlcNAc (42 µmol min^{-1} mL^{-1}), respectively. These results were in good agreement with a previous work in which we demonstrated that syntheses of LacNAc and allo-LacNAc occurring during the transgalactosylation of GlcNAc catalysed by BcβGal in aqueous media were kinetically and thermodynamically controlled, respectively [35].

Once again, systematic analysis of the dependence of rates of GlcNAc transgalactosylation on the substrate concentrations revealed plots of initial velocity versus [*o*NPG] and [GlcNAc] (5–50 mM) that followed Michaelis–Menten kinetics. These results were plotted on a Lineweaver–Burk plot. The corresponding families of the double reciprocal plots were linear and gave intersecting patterns above the *x*-axis and at the left of the *y*-axis (Fig. 2A and B). These patterns ruled out a ping pong Bi Bi mechanism. The kinetic

Table 3
Summary of kinetic constants for the transgalactosylation of GlcNAc catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures.

Substrate	K_m (mM)[a]	Dissociation constant, K_{ia} (mM)[a]	V_{max} (μmol min^{-1} mL^{-1})[a,b]	Catalytic efficiency (V_{max}/K_m) (min^{-1} mL^{-1})
*o*NPG	4.9 ± 2.1	26.1 ± 7.3	548 ± 42	0.11
GlcNAc	20.8 ± 4.0	–		0.03

[a] Obtained from Eq. (1).
[b] Initial velocities were calculated in micromoles of substrate transformed per minute and per millilitre of non diluted enzyme solution.

mechanism of BcβGal was most likely sequential, either a steady-state (Theorell-Chance or ordered) or a rapid equilibrium random Bi Bi mechanism [27]. We excluded the possibility of a rapid equilibrium random mechanism for the transgalactosylation of GlcNAc catalysed by BcβGal, given that the products of the GlcNAc transgalactosylation were formed and quantified in our system. Besides, re-plotting the slopes and intercepts against the reciprocal of the fixed substrate concentration resulted in straight lines (data not shown), further supporting the steady-state ordered Bi Bi mechanism. In addition, the probability that a Theorell-Chance system exists is very low [27]. In a steady-state ordered Bi Bi mechanism,

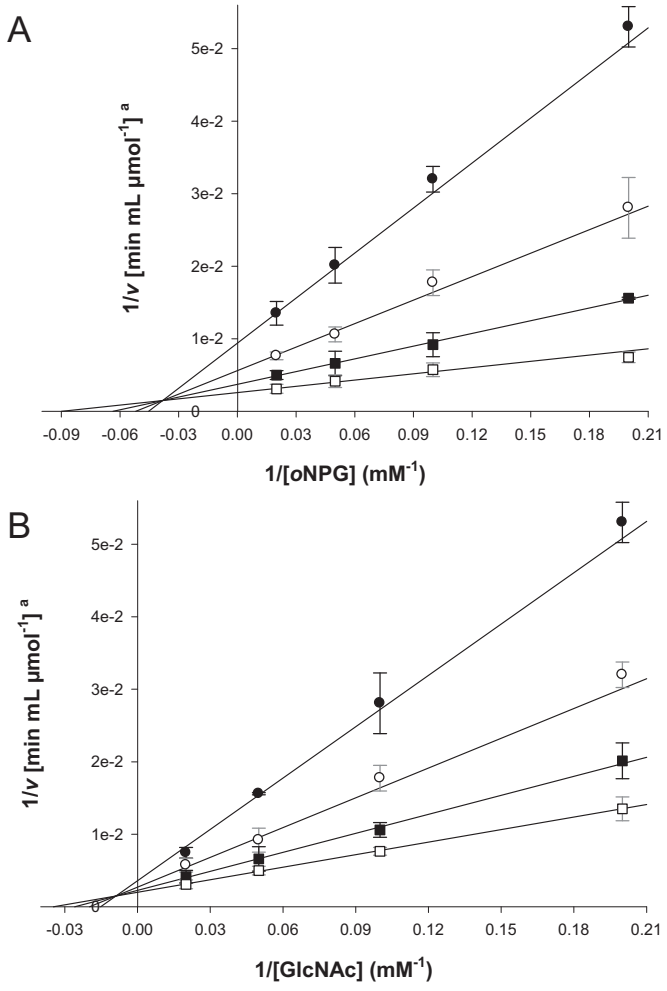


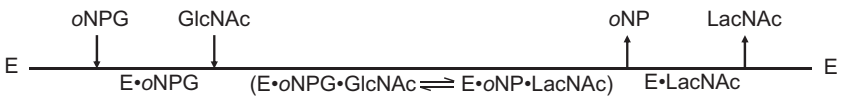
Fig. 2. Steady-state kinetics analysis of the transgalactosylation of GlcNAc catalysed by BcβGal (30 U mL^{-1}) in water/*t*-BuOH (70:30, v/v) mixtures. Double reciprocal plots of $1/v$ versus 1/[*o*NPG] with varying *o*NPG concentrations (5–50 mM) are shown (A). The concentration of GlcNAc was fixed at 5 mM (●), 10 mM (○), 20 mM (■), and 50 mM (□). Double reciprocal plots of $1/v$ versus 1/[GlcNAc] with varying GlcNAc concentrations (5–50 mM) are shown (B). The concentration of *o*NPG was fixed at 5 mM (●), 10 mM (○), 20 mM (■), and 50 mM (□). The data represent the averages of triplicate runs. [a]Initial velocities were calculated in micromoles of substrate transformed per minute and per millilitre of non diluted enzyme solution.

Lineweaver–Burk plots allow to distinguish neither the order of substrate binding nor the order of product release. Nonetheless, numerous studies dealing with the kinetic mechanism of glycosidases have shown that the order of substrate binding is in most cases as follows, regarding hydrolysis as well as transglycosylation reactions controlled by steady-state ping pong [19–23] or ordered Bi Bi [10,24–26] mechanisms: the glycosyl donor binds to the enzyme before the glycosyl acceptor. For this reason, GlcNAc was considered as the second substrate binding to the enzyme after *o*NPG. The dissociation constant (K_{ia}), Michaelis constants (K_m) and V_{max} values were calculated according to Eq. (1) and are given in Table 3. The $K_{m,oNPG}$ value (4.9 mM) was logically of the same order of magnitude as the $K_{m,app}$ values obtained by the kinetics analysis of the total transformation of *o*NPG in water (9.0 mM; see Table 1, entry 6) and in 100 mM phosphate buffer, pH 6.5 (10 mM $MgCl_2$) (3.7 mM; see Table 1, entry 7). It was also in accordance with the change in $K_{m,app}$ values observed as the concentration of GlcNAc was increased (see Table 1, entries 1–6): the lowest found for 50 mM GlcNAc was higher but close to 4.9 mM. Furthermore, the K_{ia} constant characterising the dissociation of *o*NPG from the E·*o*NPG complex was higher than the $K_{m,oNPG}$, which was confirmed by the patterns of Scatchard and Eadie-Hofstee's secondary representations (data not shown) that can be used in the case of a steady-state ordered Bi Bi mechanism [27].

3.3. Product inhibition study of the transgalactosylation of GlcNAc catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures using *o*NPG as a galactosyl donor

To confirm that the mechanism of the transgalactosylation of GlcNAc catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures using *o*NPG as a galactosyl donor is ordered and to discern the sequence of binding and release of products, product inhibition experiments were performed using *o*NP as the inhibitor of the reaction and *o*NPG as the variable substrate. GlcNAc was kept at a fixed concentration (50 mM), approximately near its K_m. The data from these experiments were screened against all the inhibition models in the "Enzyme Kinetics 2004 1.2" software. From all these possible models, the equation that specifies a noncompetitive inhibition model (Eq. (2)) was shown to best fit these data using non-linear regression, according to the analyses of error of fit given by the software. Lineweaver–Burk plots obtained for this product inhibition study are depicted in Fig. 3. The inhibition constant (K_i) for *o*NP was calculated by fitting data points to Eq. (2) and was shown to be close to 32 mM. This study of the inhibition induced by *o*NP, determined to be noncompetitive towards *o*NPG, confirmed the proposed steady-state ordered Bi Bi mechanism of the reaction. This was also consistent with the fact that this mechanism most likely involves *o*NPG and GlcNAc as the first and second substrates which bind to the enzyme, while *o*NP and LacNAc are the first and second products which dissociate from the enzyme, as shown in Scheme 2.

The crude preparation of *B. circulans* β-galactosidase Lactoles® L3 named as BcβGal in this study was provided to our group by Daïwa Kasei K.K. (Japan) in 2006 as a preparation identical



Scheme 2. Steady-state ordered Bi Bi mechanism of the GlcNAc transgalactosylation catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures, starting from *o*NPG as a galactosyl donor.

to that known as Biolacta® FN5, which has been extensively studied over the past twenty years. Mozaffar et al. first isolated and purified two β-galactosidases from Biolacta® FN5, β-galactosidase-1 (212 kDa) and β-galactosidase-2 (145 kDa) [36]. In a latter study, Vetere and Paoletti isolated from Biolacta® FN5 a third β-galactosidase, β-galactosidase-3, with a molecular mass of 86 kDa [37] and, very recently, Song et al. demonstrated the presence in Biolacta® FN5 of four immunologically homologous β-galactosidases, β-Gal-A (195 kDa), β-Gal-B (160 kDa), β-Gal-C (135 kDa) and β-Gal-D (86 kDa), exhibiting differential behaviours towards galactooligosaccharide production from lactose [38]. According to these authors, all these studies have shown that ambiguities concerning the β-galactosidases present in Biolacta® FN5 and thus in Lactoles® L3 clearly exist. Nevertheless, one fact seems to be unchanging: one of these β-galactosidases is much more active than the others towards the hydrolysis of *o*NPG. Vetere and Paoletti showed that β-galactosidase-1 had a specific activity 5- and 10-fold higher than those of β-galactosidase-2 and β-galactosidase-3, respectively, whereas the K_m values were similar for the three forms, when carrying out the hydrolysis of *o*NPG at 37 °C and pH 6.5 [37]. On the other hand, Song et al. showed that β-Gal-A exhibited a specific activity 3–4-fold higher than those of the 3 other forms β-Gal-B, β-Gal-C and β-Gal-D, and a K_m value about 2-fold lower, when conducting the hydrolysis of *o*NPG at 40 °C and pH 6.0 [38]. On the basis of these facts, the steady-state ordered Bi Bi kinetic mechanism observed for the transgalactosylation of GlcNAc catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures might be the reflect of the activity of the more active type, that is to say β-galactosidase-1 or β-Gal-A. However, further experiments including the isolation, the purification and the enzyme assays of steady-state kinetics for each different form of β-galactosidase present in Lactoles® L3 would be necessary to assess this hypothesis.

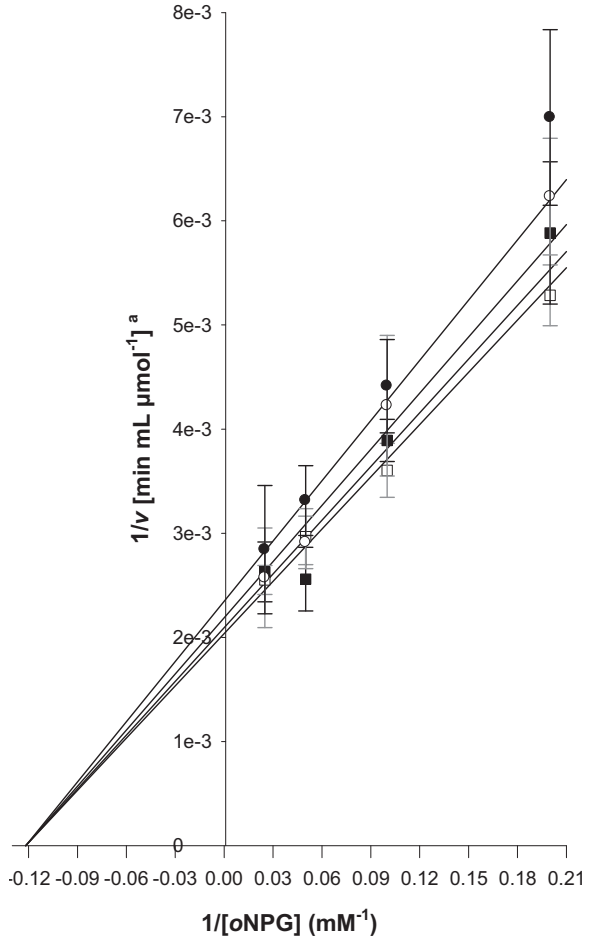


Fig. 3. Steady-state kinetics analysis of the inhibition effect induced by *o*NP on the transgalactosylation of GlcNAc catalysed by BcβGal (30 U mL^{-1}) in water/*t*-BuOH (70:30, v/v) mixtures. Double reciprocal plots of 1/v versus 1/[*o*NPG] with varying *o*NPG concentrations (5–50 mM) are shown. The concentration of GlcNAc was fixed at 50 mM and various concentrations of *o*NP were used: 0 mM (□), 1 mM (■), 2.5 mM (○), and 5 mM (●). The data represent the averages of triplicate runs. [a]Initial velocities were calculated in micromoles of substrate transformed per minute and per millilitre of non diluted enzyme solution.

Furthermore, BcβGal is a retaining β-galactosidase that is assumed to operate through a double displacement mechanism, both steps involving a transition state with a substantial oxocarbenium ion character [15–18]. For this reason, it seems difficult to understand how the steady-state ordered Bi Bi kinetic mechanism could be compatible with this known catalytic mechanism. Our hypothesis is that both *o*NPG and GlcNAc have to enter and weakly bind into the enzyme active site to generate the E·*o*NPG·GlcNAc ternary-complex before the first displacement takes place and thus before the galactosyl-enzyme intermediate is generated. Catalysis would then occur according to the known catalytic mechanism, leading to the dissociation of *o*NP and LacNAc in this order.

4. Conclusion

We have shown in this study that *t*-BuOH has various effects on the transformation of *o*NPG or lactose catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures. *t*-BuOH might thus be likened to a noncompetitive inhibitor towards *o*NPG and a competitive inhibitor towards lactose. Indeed, the use of *t*-BuOH negatively affected both the transformation velocity of *o*NPG and the affinity of BcβGal for lactose. The inhibiting properties of *t*-BuOH on *o*NPG and lactose were most likely due to the potential presence of the organic co-solvent inside the enzyme's active site, which could interfere with the displacement of the *o*-nitrophenol leaving group and prevent the entry of lactose into the active site by steric hindrance. Moreover, GlcNAc was found to act both as a substrate and an activator of the GlcNAc transgalactosylation catalysed by BcβGal in water/*t*-BuOH (70:30, v/v) mixtures, starting from *o*NPG as a galactosyl donor. This was explained by demonstrating that the reaction kinetically proceeded via a sequential steady-state ordered Bi Bi mechanism, where *o*NPG and GlcNAc bind to the enzyme as *o*NP and LacNAc leave the enzyme, most likely in this order. Very few examples of glycosidase-catalysed transglycosylation reactions have shown this type of kinetic mechanism [24,25] and our study provides for the first time the kinetic characterisation of a transglycosylation reaction catalysed by a β-galactosidase in a hydro-organic medium.

Acknowledgements

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