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Effects of Carbohydrates on the *o*NPG Converting Activity of β-Galactosidases

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Supporting Information

ABSTRACT: The effects of high concentrations of carbohydrates on the *o*-nitrophenyl β-D-galactopyranoside (*o*NPG) converting activity of β-galactosidase from *Bacillus circulans* are studied to get a better understanding of the enzyme behavior in concentrated and complicated systems in which enzymatic synthesis of galacto-oligosaccharides is usually performed. The components that were tested were glucose, galactose, lactose, sucrose, trehalose, raffinose, Vivinal GOS, dextran-6000, dextran-70 000, and sarcosine. Small carbohydrates act as acceptors in the reaction. This speeds up the limiting step, which is binding of the galactose residue with the acceptor and release of the product. Simultaneously, both inert and reacting additives seem to cause some molecular crowding, which results in a higher enzyme affinity for the substrate. The effect of molecular crowding on the enzyme activity is small compared to the effect of carbohydrates acting in the reactions as acceptors. The effects of reactants on β-galactosidases from *B. circulans*, *A. oryzae*, and *K. lactis* are compared.

KEYWORDS: *galacto-oligosaccharides, GOS, β-galactosidase, Bacillus circulans, enzyme activity, carbohydrates, crowding, galactosyl transfer, transgalactosylation, Aspergillus oryzae, Kluyveromyces lactis*

INTRODUCTION

Galacto-oligosaccharides (GOS) are widely recognized as prebiotics because they are indigestible in the human intestine and have a positive effect on the microflora in the colon.[1,2] They are commonly added to infant nutrition formula and to yogurts and drinks.[2,3]

GOS are typically produced from lactose via enzymatic synthesis with β-galactosidases.[1,3−5] A β-galactosidase preparation from *Bacillus circulans*, called Biolacta N5, is known for its high transgalactosylation activity compared to other β-galactosidases.[6,7] It produces relatively large amounts of oligosaccharides with a higher degree of polymerization compared to β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis* for example.[6,8] High GOS yields are obtained especially at high initial lactose concentrations.[6,9]

Reaction rates, equilibria, and mechanisms of biochemical reactions are usually investigated in diluted systems. In order to better understand lactose conversion in concentrated systems, where large amounts and many different types of reactants and products are present, it is essential to study the effect of these carbohydrates on GOS synthesis. Each of these reactants and products may have its influence on GOS production: they might be converted into other products or might inhibit the reaction.[5] These reactants and products influence the reaction, especially at high concentrations, and thus co-determine the yield of GOS.

Another phenomenon that might play a role in concentrated systems is molecular crowding.[10,11] The high concentration of molecules physically excludes some of the volume for other molecules. The excluded volume is the volume in a system that is occupied by molecules and cannot be occupied by the center of other molecules. This means that the effective concentration of components can be much larger than the actual concentration. Molecular crowding can affect the reaction rate, the reaction equilibrium, the enzyme activity, and the stability and can cause diffusion limitation.[12−14] Molecular crowding has been mainly studied for a better understanding of biochemical reactions in a cellular environment, but it is also relevant for enzymatic reactions under concentrated conditions.

The aim of this study is to investigate the effect of inert and reacting carbohydrates on the behavior of Biolacta N5 in concentrated systems. The effects of broad concentration ranges of several inert components (sarcosine and dextrans) and of reacting carbohydrates on the behavior of β-galactosidase from *B. circulans* during *o*NPG conversion were investigated. Because of the competitive effect between *o*NPG and other reactants (e.g., lactose), using the combination of *o*NPG with these reactants will give more insight in the mechanism than only using these reactants without *o*NPG.

The addition of reactants to the *o*NPG assay was investigated for β-galactosidases from both *A. oryzae* and *K. lactis*, to obtain a better mechanistic understanding of the activity of various β-galactosidases and to investigate the reason of the high GOS yields when using Biolacta N5.

MATERIALS AND METHODS

Materials. Lactose monohydrate (Lactochem), Vivinal GOS, and a β-galactosidase preparation from *B. circulans* called Biolacta N5 (Daiwa Kasei K. K., Japan) were gifts from FrieslandCampina (Beilen, The Netherlands). Biolacta N5 was previously found to have a total protein content of 19 ± 3%.[15] In all calculations, the total enzyme concentration was assumed to be equal to the total protein concentration, because the actual enzyme concentration is not known.

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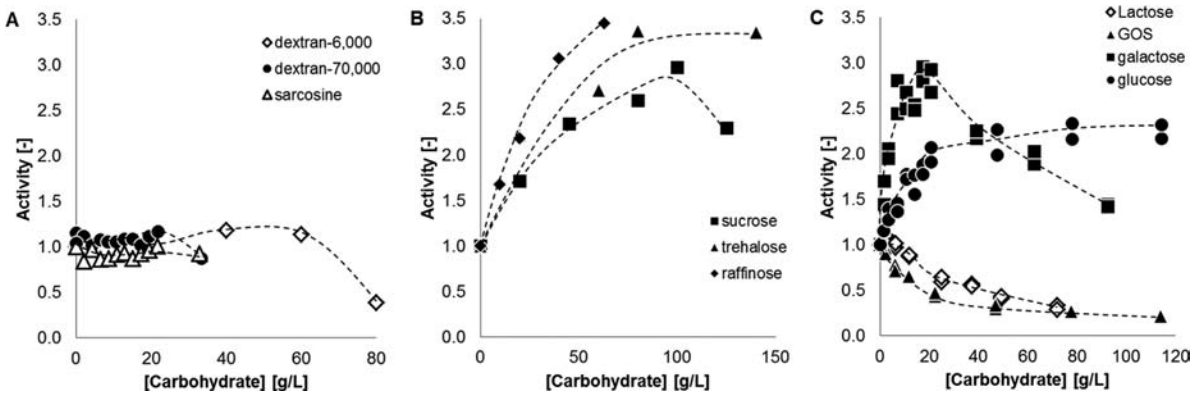

Figure 1. Initial *o*NPG converting activity of Biolacta N5 as a function of the carbohydrate concentration at 40 °C and pH 6.0 in the presence of (A) inert carbohydrates (with sarcosine and dextran-70 000 enzyme concentration during assay = 1.6 mg protein·L^{-1}; with dextran-6000, 0.8 mg protein·L^{-1}); (B) small carbohydrates (enzyme concentration during assay = 0.8 mg protein·L^{-1}); and (C) reactants (enzyme concentration during assay = 2.8 mg protein·L^{-1}). The activity is relative to the activity without added carbohydrates (lines for guidance).

β-Galactosidase from *A. oryzae* (Lactase L017P) was a gift from Biocatalysts (Nantgarw, UK). *β*-Galactosidase from *Kluyveromyces lactis* (Lactozyme) was purchased from Sigma-Aldrich (Steinheim, Germany). The enzyme concentration was calculated based on the total mass of solid and liquid taken from the preparation for *A. oryzae* and *K. lactis*, respectively.

D-(+)-Galactose, D-(+)-glucose, sucrose, D-(+)-trehalose dihydrate, D-(+)-raffinose pentahydrate, sarcosine (*N*-methylglycine), dextran-6000 and -70 000 (from *Leuconostoc* spp.), maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, sulfuric acid, sodium hydroxide, *o*-nitrophenyl *β*-D-galactopyranoside (*o*NPG), and *o*-nitrophenol (*o*NP) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate, citric acid monohydrate, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany).

McIlvaine's buffer was prepared by adding together 0.1 M citric acid and 0.2 M disodium hydrogen phosphate in the right ratio to achieve a pH of 6.0 or 8.0.

Effect of Carbohydrates on Activity. The enzyme activity measurements were adapted from Nakanishi et al.[7] A stock solution of *o*NPG in buffer with a concentration of 0.25% (w/w) was prepared. In addition, solutions of galactose, glucose, lactose, sucrose, trehalose, raffinose, sarcosine, dextran-6000, and dextran-70 000 in buffer with varying concentrations were prepared. Stock solutions of Biolacta N5 were prepared in McIlvaine's buffer of pH 6.0. The stock solution of *β*-galactosidase from *A. oryzae* was prepared by adding 37 mg of solid enzyme preparation into 50 mL of McIlvaine's buffer of pH 6.0, and the stock solution of *β*-galactosidase from *K. lactis* was prepared by adding 0.52 g of liquid enzyme preparation into 4.6 mL of McIlvaine's buffer of pH 8.0.

An Eppendorf tube with a mixture of 790 μL of 0.25% (w/w) *o*NPG solution and 189 μL of carbohydrate solution was preheated in an Eppendorf Thermomixer at 40 °C and 600 rpm for 10 min. Subsequently, 21 μL of enzyme solution was added, and the mixtures were incubated for another 10 min at 40 °C and 600 rpm. In control measurements, enzyme solution was replaced with buffer solution.

A volume of 1.0 mL of 10% (w/w) Na_2CO_3 solution was added to stop the reaction; afterward, the absorbance of *o*NP was measured at 420 nm. The *o*NP concentration was determined using the law of Lambert–Beer (ε = 4576 $M^{-1}\cdot cm^{-1}$). The carbohydrates did not affect the extinction coefficient. The *o*NP formation was found to be linear during the first 10 min of the reaction. This initial rate of *o*NP formation was expressed in mmol $min^{-1}\cdot g$ $protein^{-1}$ unless otherwise stated.

Kinetics of *o*NPG Conversion in the Presence of Carbohydrates. In order to determine the K_m and v_{max} for *o*NPG conversion with Biolacta N5 in the absence and presence of several carbohydrates, *o*NPG solutions with concentrations varying from 3.4 to 67 mM and a Biolacta N5 solution of approximately 200 mg·L^{-1} were prepared. In addition, solutions were prepared with varying concentrations of glucose, sucrose, trehalose, raffinose, and dextran-6000 in buffer. During the activity assay, 790 μL of *o*NPG solution, 189 μL of carbohydrate solution, and 21 μL of enzyme solution were incubated together for 0, 3, 5, and 10 min.

A volume of 1.0 mL of 10% (w/w) Na_2CO_3 solution was added to stop the reaction, and, afterward, the absorbance of *o*NP was measured at 420 nm. The initial rate of *o*NP formation was determined for each combination of initial *o*NPG concentration and carbohydrate concentration. The kinetic parameters K_m and v_{max} were determined with simple Michaelis–Menten kinetics:

$$v_1 = \frac{v_{max}[S_1]}{K_m + [S_1]} \quad (1)$$

where v_1 is the initial reaction rate of *o*-nitrophenol formation in mmol $min^{-1}\cdot g$ $protein^{-1}$, v_{max} is the maximum reaction rate of *o*NP formation in mmol·$min^{-1}\cdot g$ $protein^{-1}$, $[S_1]$ is the initial concentration of *o*NPG during the assay in mol·L^{-1}, and K_m is the Michaelis–Menten parameter for *o*NPG in mol·L^{-1}.

Water Activity of Carbohydrate Solutions. The water activities of solutions of sucrose, trehalose, dextran-6000, and dextran-70 000 in McIlvaine's buffer of pH 6.0 at varying concentrations were measured with an Aqualab water activity meter (model 3TE, Decagon Devices, Inc., Pullman, WA, USA) at 40 °C. This water activity meter allows measurements from 15 to 40 °C with an accuracy of ±0.003 aw. Before the water activity was measured, the solutions were kept in a water bath to be equilibrated at 40 °C. The measured carbohydrate concentrations were between 0 and 129 g·L^{-1}.

Solubility of *o*NPG in Carbohydrate Solutions. The solubility of *o*NPG in several carbohydrate solutions was determined at 23 or 40 °C. Solutions of sucrose, trehalose, and raffinose with concentrations varying between 4.2 and 33 g·L^{-1} were prepared. Subsequently, either 0.02 or 0.03 g of *o*NPG, for the solubility test at 23 or 40 °C, respectively, was dissolved in 1.0 mL of the carbohydrate solutions at a temperature of 65 °C. Afterward, the temperature was cooled to 23 or 40 °C and was constant during the night. During this, crystallization occurred.

The obtained suspensions were centrifuged in a Beckman Coulter Allegra X-22R centrifuge for 15 min at 15 500 rpm and 23 or 40 °C to remove the crystals from the supernatant. The supernatant was transferred to cuvettes, and the absorbance was measured with a spectrophotometer at 320 nm. The maximum concentration of *o*NPG that was soluble in the solutions was determined using the law of Lambert–Beer. The extinction coefficient was determined to be 2232 $M^{-1}\cdot cm^{-1}$.

Sample Preparation for Analysis of the Product Composition. Volumes of 790 μL of 0.25% (w/w) *o*NPG solution, 189 μL of 105 g·L^{-1} galactose or 108 g·L^{-1} glucose or 465 g·L^{-1} trehalose solution, and 21 μL of Biolacta N5 (200 mg solids·L^{-1}) were

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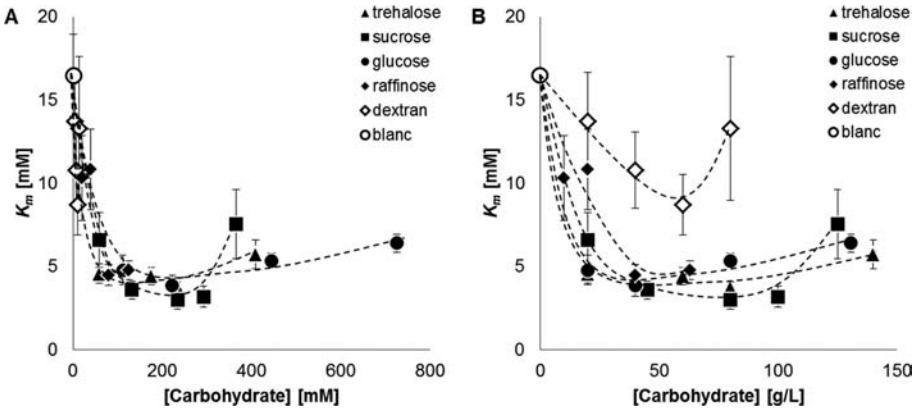

Figure 2. Michaelis–Menten constant K_m of β-galactosidase from *Bacillus circulans* for *o*NPG plotted as a function of the carbohydrate concentration, in (A) mM and (B) g·L^{-1}, at 40 °C and pH 6.0 (lines for guidance).

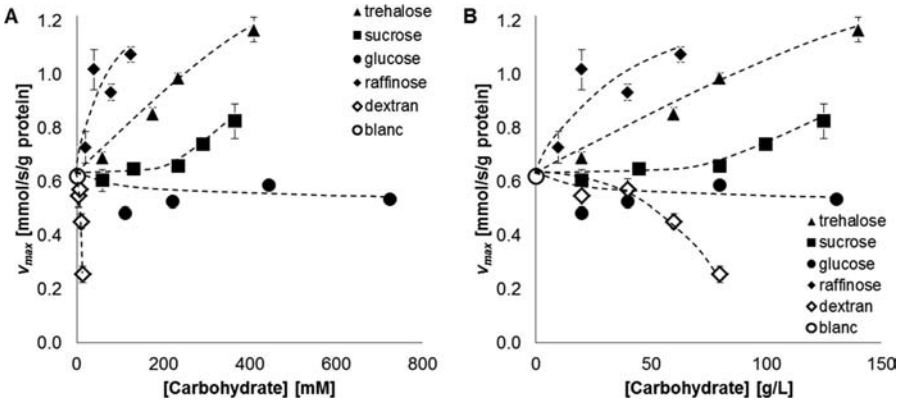

Figure 3. Maximum reaction rate of *o*NP formation v_{max} with β-galactosidase from *Bacillus circulans* plotted as a function of the carbohydrate concentration, in (A) mM and (B) g·L^{-1}, at 40 °C and pH 6.0 (lines for guidance).

incubated together at 40 °C. After 0, 10, 60, and 120 min of incubation, samples of 200 μL were taken and added to 100 μL of 5% (w/w) sulfuric acid to inactivate the enzyme. The same procedure was also carried out with reference samples in which one or more of the components were replaced with buffer solution.

Before the HPLC analysis, the enzyme was removed from the samples by filtering the samples at 14000*g* and 18 °C for 30 min using pretreated Amicon Ultra-0.5 centrifugal filter devices (Millipore Corporation, Billerica, MA, USA) with a cutoff value of 10 kDa in a Beckman Coulter Allegra X-22R centrifuge. The pretreatment of the filters consisted of two centrifugation steps: first, 500 μL of Milli-Q water was centrifuged at 14000*g* at 18 °C for 15 min, and second, the filters were placed upside down in the tube and centrifuged at 14000*g* at 18 °C for 5 min. After filtration, the samples were neutralized with 5% (w/w) sodium hydroxide.

Measurement of the Carbohydrate Composition. The filtered samples were analyzed with HPLC using a Rezex RSO oligosaccharide column (Phenomenex, Amstelveen, The Netherlands) at 80 °C. The column was eluted with Milli-Q water at a flow rate of 0.3 mL/min. The eluent was monitored with a refractive index detector.

The standards that were used for calibration of the column were lactose, glucose, galactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. Galacto-oligosaccharides up to a degree of polymerization of 7 were assumed to have the same response as the glucose-oligomers with an equal degree of polymerization. This was confirmed with mass balances.

RESULTS AND DISCUSSION

Figure 1 shows the *o*NPG converting activity of Biolacta N5 in the presence of several carbohydrates at varying concentrations. The activity was not greatly affected by the presence of sarcosine and dextran 70 000, over all tested concentrations. The activity in the presence of dextran-6000 was slightly higher than in absence of dextran-6000, but was 60% lower at a dextran-6000 concentration of 80 g·L^{-1}.

In the presence of sucrose, trehalose, and raffinose, the enzyme activity was much higher than without any additives: the activities increased up to 350%. This activity increase is similar to the activity increase of α-amylase by addition of a.o. sucrose and trehalose described by Yadav.[16] In the presence of lactose and Vivinal GOS, the *o*NPG converting activity decreased with increasing lactose or Vivinal GOS concentration. This may be due to the competition between *o*NPG and lactose or the oligosaccharides present in Vivinal GOS, because both are substrates for the enzyme.

The products galactose and glucose have a strong positive effect on the *o*NPG converting activity: the activities increased up to 300% and 200%, respectively. Remarkable is the high activity at low galactose concentrations and in the presence of glucose. Galactose and glucose are usually recognized to be inhibitors for β-galactosidases,[17−20] but galactose and glucose here strongly promoted the conversion of *o*NPG into *o*NP. At galactose concentrations above 30 g·L^{-1}, the activity decreased again with increasing galactose concentration, which might be a sign of dominance of inhibition.

Kinetics of *o*NPG Conversion in the Presence of Carbohydrates. The initial rates of *o*NP formation in the presence of glucose, sucrose, trehalose, raffinose, and dextran-6000 were measured (see Figure S1 in the Supporting

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Information). The data were fitted to the Michaelis–Menten model to obtain the kinetic parameters K_m and v_{max}, which are shown in Figures 2 and 3, respectively.

Figure 2 shows that the Michaelis–Menten constant K_m decreased with the addition of carbohydrates. On a molar concentration scale, this decrease was more or less independent of the type of carbohydrate added. However, on a mass concentration scale, dextran affected the K_m less than smaller carbohydrates, due to its larger molecular weight. The smaller carbohydrates affected the K_m all in a similar way.

Below carbohydrate concentrations of approximately 300 mM, the K_m decreased with increasing carbohydrate concentration, which implies that the affinity of the enzyme for the substrate *o*NPG increased with the addition of these carbohydrates. At higher carbohydrate concentrations, the K_m increased again slightly with further increase of the carbohydrate concentration.

Figure 3 shows more complex effects on v_{max} for each of the tested carbohydrates (both on a molar concentration scale and on a mass concentration scale). v_{max} did not change significantly with the glucose concentration, but in the presence of sucrose, trehalose, and raffinose, the v_{max} increased strongly with increasing carbohydrate concentration, with the largest effect for raffinose and the smallest for sucrose. Dextran-6000 had a negative effect on v_{max}: it decreased with increasing dextran-6000 concentration.

Characteristics of Reaction Medium. To exclude effects by the medium from causing changes in enzyme activity and enzyme kinetics with the addition of carbohydrates, the water activity and the solubility of *o*NPG in the carbohydrate solutions were measured.

Water Activity. The water activities of solutions of sucrose, trehalose, dextran-6000, and dextran-70 000 in buffer were measured at 40 °C (see Figure S2 in the Supporting Information). When no carbohydrates were present, the water activity was approximately 0.994. The fact that the water activity is not 1 at carbohydrate concentration 0 is due to the presence of the buffer. The water activities of each of the carbohydrate solutions were found to show only a very slight decrease from approximately 0.994 to 0.986 over a concentration range up to 130 g·L^{-1}. This small decrease cannot explain the large changes in enzyme activity and enzyme kinetics. Gosling et al.[21] found earlier that oligosaccharide levels were not affected by even larger changes in water activity.

Solubility of oNPG in Carbohydrate Solutions. The addition of carbohydrates could lead to a shift in the distribution of *o*NPG molecules between being free in solution and being bound to the enzyme, since the associated state will have a lower overall volume than the dissociated state. That would mean that with the addition of carbohydrates the *o*NPG molecule prefers to leave the crowded environment and prefers to bind to the enzyme, which could result in a decrease in K_m and an increase in the enzyme activity. If this is the case, the solubility of *o*NPG is expected to decrease with addition of carbohydrates when no enzyme is present. The solubility of *o*NPG in buffer was determined to be 15 ± 1 and 21 ± 0 g·L^{-1} at 23 and 40 °C, respectively (see Figure S3 in the Supporting Information). The addition of sucrose, trehalose, and raffinose in the tested concentration ranges did not change the solubility, which means that the changes in enzyme activity and enzyme kinetics were not caused by a change in the solubility of *o*NPG.

Since the water activity and the solubility of *o*NPG do not change with increasing carbohydrate concentration, the characteristics of the reaction medium can be excluded as cause of the changes in enzyme activity and enzyme kinetics.

Characteristics of the Enzyme. Since the characteristics of the reaction medium do not change with the addition of the carbohydrates, we also investigated the effect of the carbohydrates on the enzyme itself.

In general, *o*NPG conversion with *β*-galactosidases takes place in two steps. First, an enzyme–galactose complex is formed and *o*NP is released, and second, the galactose is released since water is used as the acceptor molecule; hydrolysis takes place. However, in the presence of carbohydrates, transglycosylation might take place. The reaction mechanism that we propose is shown in Figure 4. Instead of water, the carbohydrates may also be used as an acceptor molecule and form a product together with the galactose unit that is released from the enzyme–galactose complex. A similar phenomenon was observed by Gosling et al.[21] during lactose conversion in the presence of sucrose. In this case, the product should have a degree of polymerization that is larger than the carbohydrate added. To test this hypothesis, we measured the product composition during *o*NPG conversion with Biolacta N5 in presence of galactose, glucose, and trehalose. The results are shown in Figure 5.

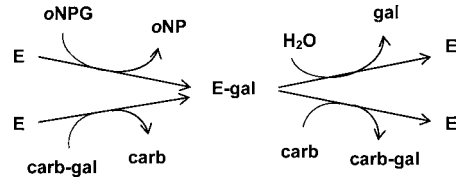

Figure 4. Schematic representation of the reaction mechanism of *β*-galactosidase from *Bacillus circulans*. Abbreviations: E, enzyme; *o*NPG, *o*-nitrophenyl *β*-D-galactopyranoside; *o*NP, o-nitrophenol; E-gal, enzyme–galactose complex; gal, galactose; carb, carbohydrate; carb-gal, oligosaccharide that consists of one galactose unit attached to another carbohydrate.

During the conversion of *o*NPG without any added carbohydrates, a slight decrease in *o*NPG concentration and a slight increase in galactose concentration were detected. When galactose, glucose, or trehalose was added, much more *o*NPG was converted, which is in agreement with the increases in activity (Figure 1). In addition, disaccharides were formed with addition of galactose and glucose, whereas trisaccharides were formed in the presence of trehalose. This confirms our hypothesis that the added carbohydrates participate in the reaction, and removing galactose from the active center, frees up the enzyme for faster *o*NPG conversion.

Some carbohydrates that do not contain a galactose moiety can act only as acceptor and thus will speed up the overall reaction by freeing up the active center; other sugars (such as galactose itself) may also act as donor, and in this case both competitive inhibition and the acceptor-acceleration take place. In this case, one may expect the optimum as was observed in Figure 1C.

Changes in K_m and v_{max} Explained. The increase in affinity caused by the addition of carbohydrates may be explained by molecular crowding. A similar K_m decrease was reported before by Jiang and Guo[22] for addition of dextrans to isochorismate synthase. Yadav[16] described an activity increase of *α*-amylase by addition of a.o. sucrose and trehalose, which was due to molecular crowding. The association of the enzyme with *o*NPG probably results in a volume reduction, which is

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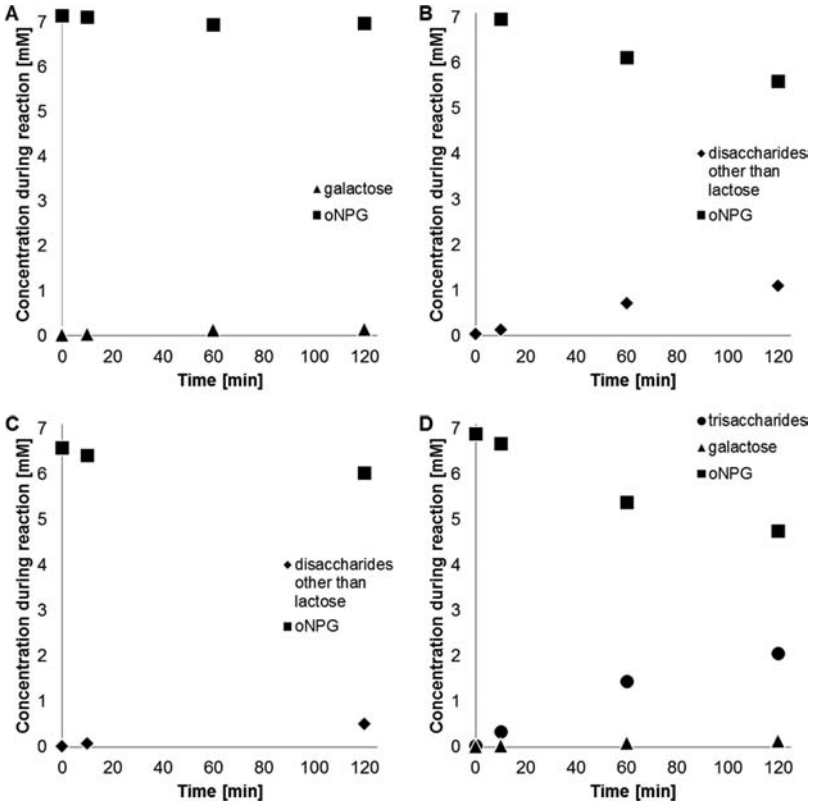

Figure 5. *o*NPG and carbohydrate profiles during *o*NPG conversion at 2.0 g·L^{-1} with 0.8−0.9 mg·L^{-1} Biolacta N5 at 40 °C and pH 6.0 in the presence of (A) no added carbohydrates, (B) 20 g·L^{-1} galactose, (C) 20 g·L^{-1} glucose, and (D) 88 g·L^{-1} trehalose. (The reaction was followed for two hours (whereas the activity assay is 10 min) to find larger differences in breakdown or formation of the components.)

thermodynamically favorable in a crowded environment.[10,23] Besides, if this enzyme is active as a folded protein, crowding will stabilize the folded proteins.[10,23]

The smaller carbohydrates have a stronger effect on a mass concentration scale on the K_m than dextran, which is inert. This may be explained by dextran having a smaller total excluded volume per gram than the small carbohydrates, although the total volume of the carbohydrates is equal in each case. Figure 6 shows the calculated total excluded volume for various concentrations of added carbohydrates. The calculations were carried out assuming the carbohydrate and the substrate to be spheres with radii R_c and R_s, with $R = 8.26 \times 10^{-1} \times MW^{1/3}$ nm (MW in kD), similar to the approach by Batra et al.[24] for dextran. The total excluded volume is equal to the number of carbohydrate molecules multiplied by the excluded volume of each carbohydrate molecule with radius $R_c + R_s$, since the center of the substrate cannot enter there. This results in a larger total excluded volume when a certain mass of small carbohydrates is present compared to when the same mass of large carbohydrates is present.[11,12] Therefore, the effect of molecular crowding in the case of small carbohydrates on the K_m is larger than in the case of dextran. In addition, the interactions of the small carbohydrates might also play a role in the reaction.

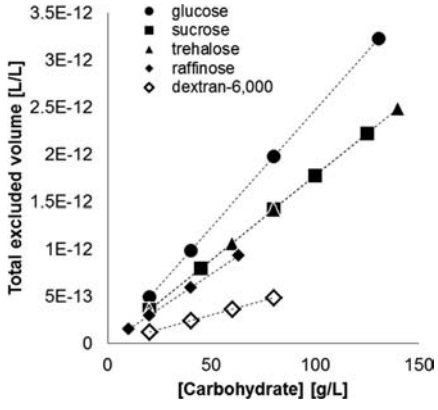

Figure 6. Calculated total excluded volume for substrate molecules as a function of the carbohydrate concentration. The total excluded volume is the amount of carbohydrate molecules multiplied with the excluded volume of each carbohydrate molecule. Carbohydrate and substrates are assumed to be spherical molecules with radii R_c and R_s, with $R = 8.26 \times 10^{-1} \times MW^{1/3}$ nm (MW in kD). The excluded volume of each carbohydrate molecule has a radius of $R_c + R_s$.

The slight increase of K_m at higher carbohydrate concentrations might be a result of diffusion limitation, because diffusion is slower in crowded systems, and thus the frequency of the encounters between enzyme and substrate is reduced.[10,23]

The maximum rate of *o*NP formation (v_{max}) was higher in the presence of sucrose, trehalose, or raffinose than with dextran mainly because these carbohydrates are acceptors in the reaction. They cause a quick release of the enzyme (from the enzyme−galactose complex), and the enzyme is free in solution again, so that another *o*NPG molecule can form a complex with the enzyme. Dextran is inert and cannot be used as an acceptor molecule.

Since an increase in v_{max} was found with increasing carbohydrate concentration, the attachment of an acceptor molecule (either water or a carbohydrate molecule) is supposed

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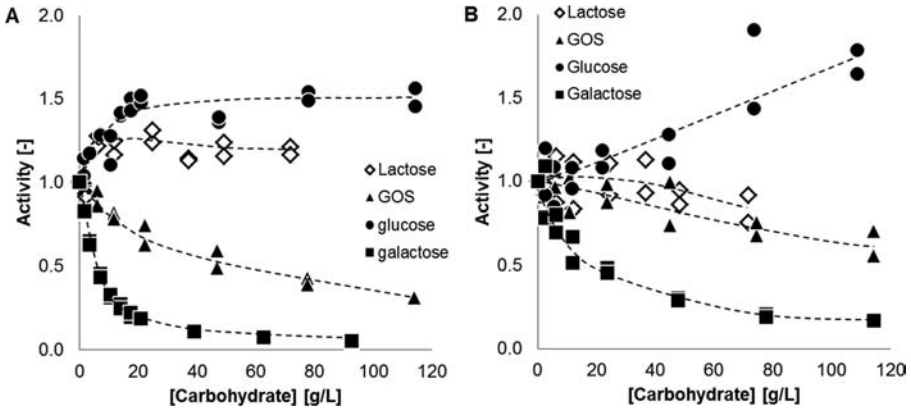

Figure 7. Initial *o*NPG converting activity of *β*-galactosidase from (A) *Aspergillus oryzae* at 15 mg solids·L^{-1}, 40 °C, and pH 6.0 and (B) *Kluveromyces lactis* at 144 mg enzyme preparation·L^{-1}, 20 °C, and pH 8.0, as a function of the carbohydrate concentration in presence of various reactants (lines for guidance).

to be the rate-limiting step. The reaction is accelerated when the carbohydrate concentration is increased. This implies that water is a very poor acceptor molecule, as the molar water content is still very high compared to the carbohydrate concentration and water molecules are very mobile. The affinity of the enzyme−galactose complex for the specific carbohydrate molecules therefore has to be much higher than the affinity for a water molecule.

The addition of glucose does not change v_{max}. According to Figure 5, the lowest conversion of *o*NPG was found with glucose among the tested carbohydrates. This can be explained by glucose acting as an uncompetitive inhibitor.[18,25] If glucose inhibits the reaction while also acting as an acceptor, these two effects may cancel out each other. Next to glucose, sucrose is also an uncompetitive inhibitor.[25] However, the inhibition constant of sucrose is higher than that of glucose,[25] which means that the affinity for sucrose (as an inhibitor) is lower than for glucose. The inhibiting effect can explain the v_{max} caused by glucose being lower than that of sucrose. Raffinose and trehalose do not have any inhibiting effect.

The presence of dextran decreased v_{max} at high concentrations, which may be due to diffusion limitations.

Overall, the effect of carbohydrates acting in the reaction as acceptors, represented by a change in v_{max}, is large compared to the effect of molecular crowding on the enzyme activity, represented by a decrease in K_m.

Mechanistic Understanding of *β*-Galactosidase Activity. For a better mechanistic understanding of the activity of *β*-galactosidases, the *o*NPG converting activity of *β*-galactosidases of *A. oryzae* and *K. lactis* was measured in the presence of small carbohydrates. The results are shown in Figure 7.

Figure 7 shows, together with Figure 1C, that the reactants (lactose, oligosaccharides, galactose, and glucose) affect the three enzymes in very different ways. In production systems with *β*-galactosidase of *B. circulans* with a high lactose concentration, where lactose is converted into GOS, the competition between lactose and Vivinal GOS for association with the enzyme is advantageous: the enzyme has a high affinity for these components and will form products with a higher degree of polymerization. The latter was previously observed by Boon et al.[6] The decrease in activity in the presence of galactose does not hinder the reaction, since hydrolysis is limited in concentrated solutions, and thus the galactose concentration remains low.

The *o*NPG converting activity of *β*-galactosidase of *A. oryzae* (Figure 7A) is (slightly) higher in the presence of lactose and glucose, but lower in the presence of Vivinal GOS and galactose, with the lowest activity in the presence of galactose. The higher activity in the presence of lactose implies that lactose is used as an acceptor molecule instead of a donor molecule: the affinity of the enzyme for *o*NPG is much higher than for lactose, which will not be able to displace *o*NPG. We expect glucose to act as an acceptor as well. Galactose inhibits the enzyme already at low concentrations, which is in agreement with the conclusion by Boon et al.[6] that galactose is an inhibitor.

The *o*NPG converting activity of *β*-galactosidase of *K. lactis* (Figure 7B) showed similar trends. Vivinal GOS reduced the activity. Lactose does not affect the activity much, but it probably acts simultaneously as an acceptor and as a donor. As with *β*-galactosidase from *A. oryzae*, glucose will be an acceptor; galactose once more acts as an inhibitor.[6] Since this enzyme shows much more hydrolysis activity, product inhibition by galactose would probably hinder the production of GOS even at high lactose concentrations.

Previously it was shown that Biolacta N5 was a productive enzyme preparation in terms of oligosaccharide yield compared to other *β*-galactosidases.[6,7] This can now be explained by the much higher affinity for lactose and the suitability of glucose and galactose as acceptor molecules. Besides, galactose is not inhibiting the enzyme that strongly. These mechanistic differences are the reason that the enzyme from *B. circulans* shows much higher production rates of GOS than the *β*-galactosidases of *A. oryzae* and *K. lactis* under comparable conditions.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: Measured initial rates of *o*NP formation in the presence of various carbohydrates including their fit with Michaelis−Menten kinetics. Figure S2: Water activity of various carbohydrate solutions. Figure S3: Solubility of *o*NPG in various carbohydrate solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

GOS, galacto-oligosaccharides; *o*NPG, *o*-nitrophenyl β-D-galactopyranoside; *o*NP, *o*-nitrophenol

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