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Enzymatic synthesis of galactosyl–xylose by *Aspergillus oryzae* β-galactosidase

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Abstract

In aqueous medium, the reaction catalyzed by *Aspergillus oryzae* β -galactosidase with *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in the presence of an acceptor leads to the synthesis of transglycosylation compounds in addition to the hydrolysis products (ONP and galactose). Our goal was to develop a simple system for the synthesis of galactosyl–xylose, a disaccharide of possible application to diagnostics. To maximize synthesis yields, we have studied the effect of several conditions: increase of acceptor concentration (0.05–2.7 M xylose), organic co-solvents (dimethylformamide, acetone) and reaction time.

In the absence of co-solvents ONPG was completely consumed in 2 h; with 0.5 M xylose the maximum yield of galactosyl–xylose (16%) was attained at 60 min, while with 2.7 M xylose the yield reached 21%. Both co-solvents tested decreased the kinetics of ONPG convertion into products and 50% (v/v) dimethylformamide was deleterious to the synthesis. However, in 50% (v/v) acetone the synthesis yield was 12% and interestingly, the proportion of transglycosylation with respect to the reacted substrate was higher than in buffer.

The synthesis of galactosyl–ethyleneglycol was also studied; it was achieved with extremely high yield and no detectable hydrolysis products. This proves that other acceptor alcohols can be preferred over water in some conditions.

Keywords: β-Galactosidase; Galactosyl–xylose; Enzymatic synthesis; Galactosides

1. Introduction

The synthesis of oligosaccharides is currently receiving a great deal of attention owing to their important roles in many biological processes [1,2]. The regioselective synthesis of carbohydrate derivatives by chemical methods is complicated: it requires several protection and deprotection steps because of the presence of multiple hydroxyl groups of similar reactivity [3–5]. As an alternative, biocatalytic approaches employing isolated enzymes such as glycosyltransferases and glycosidases, or engineered whole cells, are powerful strategies complementary to chemical methods alone. The use of glycosyltransferases offers regioselective transfer and high glycosylation yields. However, they require nucleotide donors that are costly and have to be generated in situ. On the other hand, glycosidase enzymes, in the presence of an acceptor alcohol or saccharide capable of trapping the glycosyl intermediate, can be exploited for glycoside or oligosaccharides synthesis. The advantage

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of glycosidases is that they are widely available, robust, and relatively tolerant to organic solvents, which makes them suitable for synthetic purposes. However, in the presence of water as a competing nucleophile the synthesis yield is generally low. To enhance the synthesis yield, high concentrations of acceptor or the inclusion of organic co-solvents has been reported to be successful in some cases [1,4–7].

Galactosyl–xylose is a disaccharide of potential application in diagnostics as a substrate for β-galactosidase. Here we report the synthesis of galactosyl–xylose both in aqueous medium and in the presence of co-solvents using β-galactosidase from *Aspergillus oryzae*. The influence of the acceptor concentration and the synthesis of galactosyl–ethyleneglycol was also studied.

2. Materials and methods

2.1. Materials

O-Nitrophenyl-β-D-galactopyranoside, β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) from *A. oryzae*, galactose, xylose, 6-*O*-β-D-galactopyranosyl-D-galactose (β-D-Gal-[1→6]-D-Gal), 3-*O*-β-D-galactopyranosyl-D-arabinose (β-D-Gal-[1→3]-D-Ara), 4-*O*-(3-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-D-galactopyranose (α-D-Gal-[1→3]β-D-Gal [1→4]-D-Gal), 3-*O*-(4-*O*-[3-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl]-α-D-galactopyranosyl)-D-galactopyranose (α-D-Ga1-[1→3]-β-D-Gal-[1→4]-α-D-Ga1-[1→3]-D-Gal), acetone, dimethylformamide (DMF) and acetonitrile were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Enzyme activity

The activity of β-galactosidase from *A. oryzae* was assayed at room temperature using ONPG as a substrate. A suitably diluted enzyme solution was added to 25 mM ONPG in 50 mM sodium acetate buffer, pH 5.5. The rate of formation of free *O*-nitrophenol (ONP) was recorded spectrophotometrically at 405 nm using a 1 cm path length cuvette. One unit of enzyme activity (U) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per minute in the conditions defined above. Enzymatic activity was expressed as EU per ml.

2.3. Synthesis of galactosyl–xylose

One milliliter of enzyme solution (10 EU/ml) was added to 10 ml of a 50 mM ONPG solution in 50 mM acetate buffer pH 5.5 containing different xylose concentrations (0.05 M; 0.5 M; 2.7 M). The mixture was incubated at room temperature for 2 h and aliquots were taken at regular intervals. The reaction was stopped by heating at 100 °C for 5 min and the samples were analyzed for carbohydrates as described below.

The synthesis of galactosyl–xylose was also performed in the same conditions except for the presence of 50% (v/v) buffer- dimethylformamide and 50% (v/v) buffer-acetone. In these cases the synthesis reaction was monitored for 5 h.

2.4. Synthesis of galactosyl–ethyleneglycol

One milliliter of enzyme solution (10 EU/ml) was added to 10 ml of a 50 mM ONPG solution in 50 mM sodium acetate buffer pH 5.5 containing 50% (v/v) ethyleneglycol and incubated at room temperature for 4 h. Aliquots were taken at regular intervals, the reaction was stopped by heating at 100 °C for 5 min and the samples were analyzed for carbohydrates as described below.

2.5. Assay of carbohydrates

The samples were deproteinized by the following treatment: 200 μl of double distilled water and 800 μl of acetonitrile were added to 200 μl of each sample, the mixture was centrifuged for 5 min and filtered through 0.5 μ pore filters. The saccharides were analyzed in a Waters High-Performance Liquid Chromatography (HPLC) system, equipped with refractive index detector. A Shodex Asahiapak NH_2 P50 4E , 4.6 mm i.d. × 250 mm length column was used at 25 °C with acetonitrile–water (75:25) as the mobile phase and a flow rate of 1 ml per ml.

The following purified glycosides commercially available were used as standards of galactobioses, galactotrioses and galactotetraoses respectively: β-D-Gal-[1→6]-D-Gal, α-D-Gal [1→3]-β-D-Gal [1→4]-D-Gal, and α-D-Gal-[1→3]-β-D-Gal-[1→4]-α-D-Gal-

[1→3]-D-Gal. Galactosyl-arabinose (β-D-Gal-[1→3]-D-Ara) was used as a structurally related standard of galactosyl–xylose, which was not commercially available. It is important to mention that our chromatographic system does not separate stereoisomers such as glucose and galactose.

3. Results

3.1. Synthesis of galactosyl–xylose

In aqueous medium, when ONPG is used as a galactose donor and xylose as an acceptor, the reaction catalyzed by β-galactosidase from *Aspergillus oryzae* is expected to yield several transglycosylation compounds in addition to the two hydrolysis products (ONP and galactose). As the galactose released during the hydrolysis of ONPG can also act as acceptor, galactobiose and eventually galactotriose and galactotetraose could also be formed. In general, microbial β-galactosidases produce β 1→6 linked galactosides during transglycosylation of ONPG, due to the high reactivity of the primary hydroxyl group acceptor. However, products with other linkages (1→3 or 1→4) could also be formed depending on the structure of the acceptor [8–10].

A typical HPLC elution pattern of the reaction mixture is shown in Fig. 1 in which one additional peak is detected with respect to the HPLC traces of the starting materials and the hydrolysis products of ONPG. This new peak showed the same retention time (8.5 min) as the galactosyl–arabinose standard (Table 1), pointing out to the presence of the galactosyl–xylose product. There were no peaks with retention time close to the galactobiose standard. In addition, the presence of galactotrioses or tetraoses was not detected With the chromatographic column used in this work and the available standards we are not able to determine the type of linkage between the galactose and xylose, or if a mixture of galactosyl–xylose products with different linkages are formed.

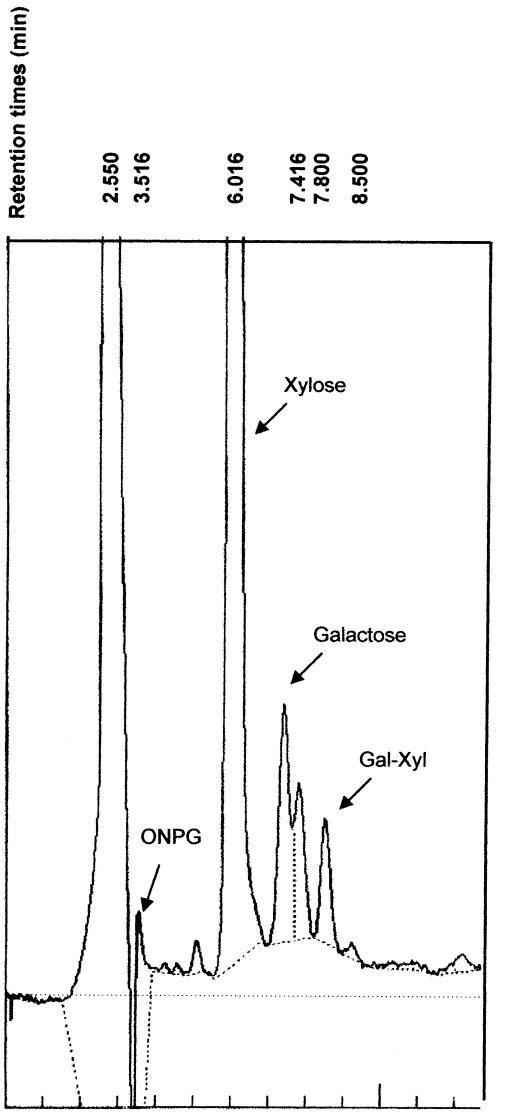

Fig. 1. HPLC chromatogram of saccharide mixtures formed from ONPG and xylose by *A. oryzae* β-galactosidase.

Table 1
Retention times of mono-and oligosaccharides used as standards in HPLC analysis in the synthesis of galactosyl–xylose

Saccharide	Retention time (min)
ONPG	3.5 ± 0.2
Xylose	60 ± 0.1
Galactose	7.4 ± 0.1
β-D-Gal [1→3] Ara	8.4 ± 0.1
β-D-Gal [1→6] Gal	11.8 ± 0.1
α-D-Gal [1→3] β-D-Ga1 [l→4] Gal	15.0 ± 0.1
α-D-Gal [l→3]-β-D-Gal [1→4]-α-D-Gal [1→3]-D-gal	23.7 ± 0.2

The performance of the reaction was expressed in two ways: by the “synthesis yield”, and by the “transglycosylation rate”. The synthesis yield was defined as

the amount of product obtained (galactosyl–xylose), as a percentage of the total amount of galactosyl donor (ONPG) initially applied. The "transglycosylation rate" was defined as the amount of product formed (galactosyl–xylose), as a percentage of the galactosyl donor (ONPG) converted (both by hydrolysis and transglycosylation). While the synthesis yield evidences the overall yield of the synthetic reaction of interest with respect to the total amount of donor applied, the transglycosylation rate shows the proportion of this reaction towards the hydrolysis and other possible transglycosylation reactions, and it is useful to characterize the influence of the reaction conditions in the process of transglycosylation.

3.2. Effect of acceptor concentration

When the synthesis of galactosyl–xylose was performed in aqueous medium using ONPG as the galactosyl donor and xylose as the acceptor, the maximum yield of galactosyl–xylose produced was achieved within 30–60 min of reaction. Increasing the concentration of acceptor (xylose) did not significantly influence the kinetics of ONPG convertion, in all cases more than 80% of the initial ONPG was converted after 60 min of reaction time.

Table 2
The effect of acceptor concentration on the synthesis yield and transglycosylation rate in aqueous medium

Xylose concentration (M)	Time of max. synthesis yield (min)	Synthesis yield (%)	Transglycosylation rate (%)
0.05	30	12	12
0.5	60	16	22
2.7	60	21	25

On the other hand, the synthesis yield grew from 12 to 21% as the acceptor concentration increased from 0.05 to 2.7 M (Table 2, Fig. 2); the transglycosylation rates were slightly higher than the synthesis yields and increased with the concentration of xylose (Table 2).

3.3. Effect of co-solvents on the synthesis of galactosyl–xylose

The presence of either acetone or DMF, 50% (v/v), markedly reduced the rate of convertion of ONPG from close to 100% in 2 h in the absence of co-solvents to less than 35%. The presence of acetone (50% (v/v))

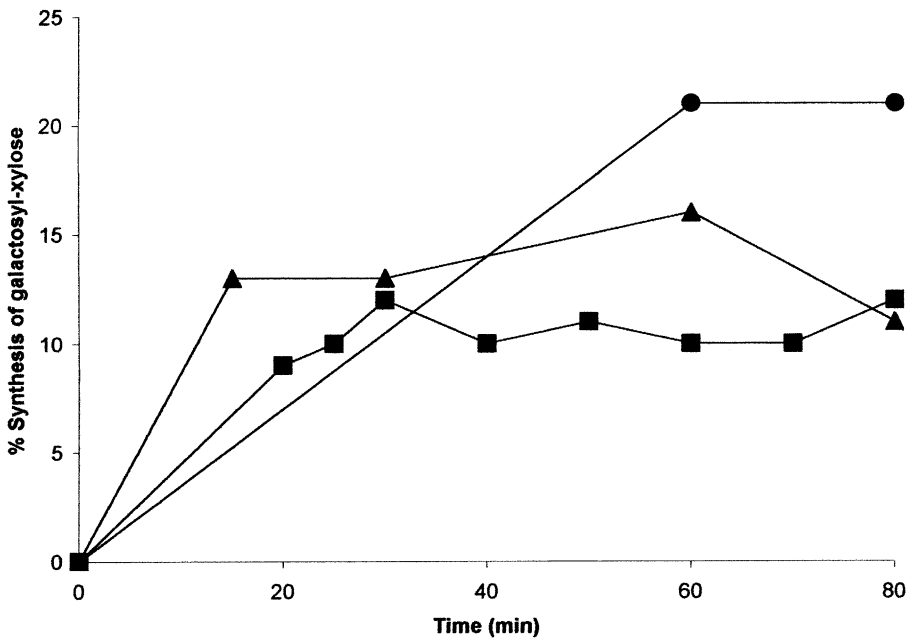

Fig. 2. Synthesis yield of galactosyl–xylose in aqueous medium: (■) 0.05 M xylose; (▲) 0.5 M xylose; (●) 2.7 M xylose.

Table 3
The effect of co-solvents on synthesis yields and transglycosylation rates[a]

Co-solvent	Time of maximum synthesis yield (min)	Synthesis yield (%)	Transglycosylation rate (%)
None	60	16	22
50%DMF	340	4	11
50%Acetone	90	12	45

[a] All reactions were carried out with 0.5 M xylose concentration.

diminished the synthesis yield of galactosyl–xylose from 16 to 12%, but interestingly, the transglycosylation rate was doubled (Table 3), so that 45% of the enzyme-bound galactose was transferred to the xylose molecule. In 50% (v/v) DMF the synthesis yield decreased to 4%, and the time at which maximum yield was achieved increased to 340 min. Again the transglycosylation rate was higher (11%) than the synthesis yield.

3.4. Synthesis of galactosyl–ethyleneglycol

In the presence of 50% (v/v) ethyleneglycol, the convertion of ONPG was slower than in aqueous medium; after 240 min only 70% of the ONPG was converted. For the synthesis of galactosyl–ethyleneglycol a maximum yield was never reached as the synthesis yield increased throughout the experiment. Moreover, the transglycosylation rate was 100% at every time point, which means that free galactose did not appear at all. This fact clearly indicates that ethyleneglycol is a better acceptor than xylose and water in the experimental conditions described (Table 4).

Table 4
Synthesis yield and transglycosylation rate of galactosyl-ethyleneglycol[a]

Time (min)	Synthesis yield (%)	Transglycosylation rate (%)
0	0	0
10	8	100
30	16	100
40	22	100
70	38	100
180	58	100
240	70	100

[a] Ethyleneglycol was present at 50% (v/v) in all cases.

4. Discussion

β-Galactosidases from different sources are known to catalyze transglycosylation reactions by means of both equilibrium and kinetically controlled processes. The enzyme from *Aspergillus oryzae* is one of the most stable commercially available β-galactosidases and also exhibits a significant transgalactosylase activity [11]. Our goal was to develop a very simple system for the synthesis of galactosyl–xylose, as a substrate for β-galactosidase. At this stage we have focused on the optimization of the conditions of the synthetic reaction, and the characterization of the type of linkage of the synthesis products will be the subject of further studies.

The concentration of the acceptor and the presence of co-solvents are key factors that determine the transglycosylation rate, and in this work we have studied both effects. These factors reduce the water activity and tend to favor the transglycosylation reaction over the hydrolysis reaction. While the presence of either DMF or acetone (50% (v/v)) significantly reduced the ONPG convertion (Fig. 3a), increasing the xylose concentration to 2.7 M (41% (w/v)) did not have a similar effect. Increasing the acceptor concentration did favor the synthesis reaction as well as the transglycosylation rates (Table 2) but at the expense of very high excess of acceptor. So, most of the xylose remained unreacted. This fact should be taken into consideration when selecting optimal synthesis conditions. On the other hand, the transglycosylation rate for galactosyl–xylose improved in the presence of 50% (v/v) acetone, but the synthesis yield did not (Table 3). This could be due to the fact that organic co-solvents can modify the conformation of the enzyme's active site, reducing its activity but making it more prone to accepting a larger molecule than water as the galactosyl acceptor. However, even if the overall effect of co-solvents was not favorable, in the presence of acetone the synthesis product is more stable.

In conclusion, synthesis of galactosyl–xylose could be better achieved in an aqueous medium, which favors the activity and stability of the enzyme. The impressive synthesis yield of galactosyl–ethyleneglycol demonstrates that acceptor alcohols other than water and xylose can be preferred in some conditions.

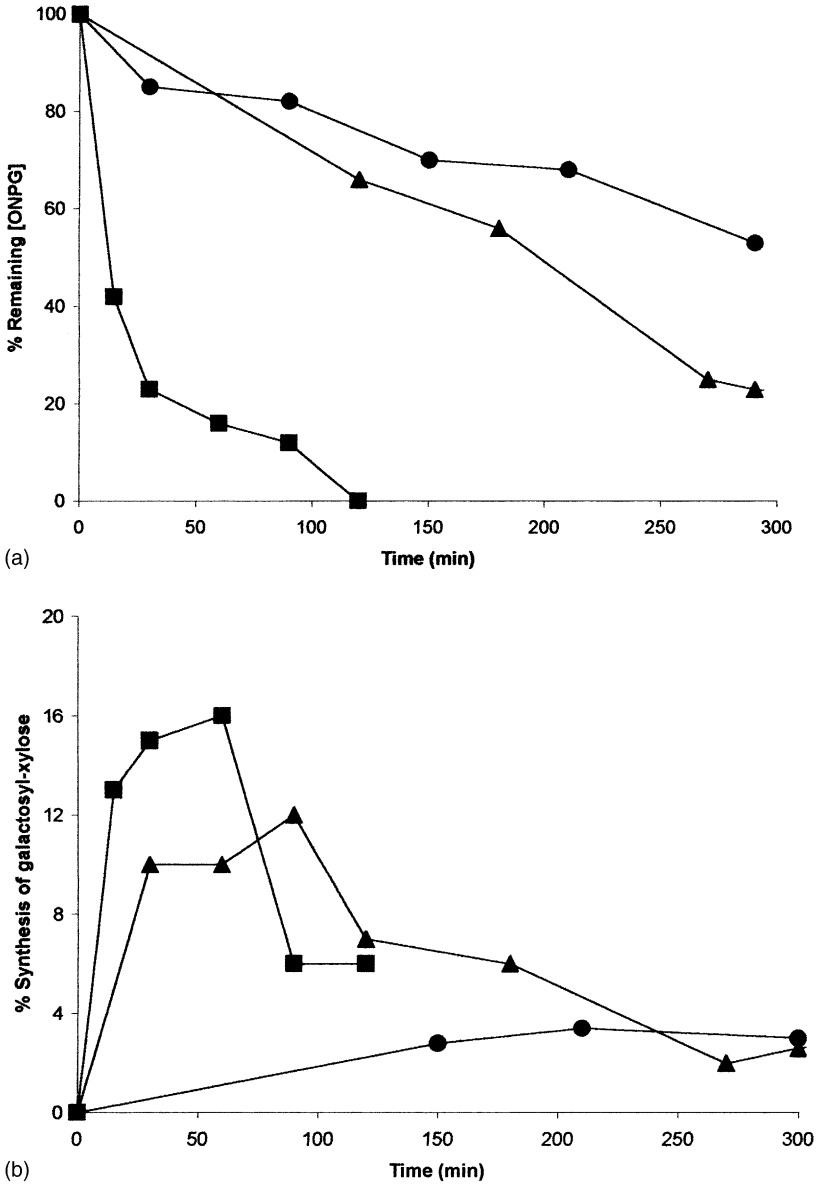

Fig. 3. Synthesis of galactosyl–xylose in the presence of co-solvents (a) Rate of ONPG convertion: (■) without co-solvent; (●) DMF 50% (v/v); (▲) acetone 50% (v/v), (b) synthesis yield of galactosyl–xylose: (■) without co-solvent; (●) DMF 50% (v/v); (▲) acetone 50% (v/v).

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