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Enzymatic synthesis of three *p*NP-α-galactobiopyranosides: application of the library of fungal α-galactosidases

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Abstract

The regioselectivity of the transglycosylation reaction catalyzed by extracellular α-galactosidases from filamentous fungi was studied using *p*-nitrophenyl α-D-galactopyranoside. Regioisomers of *p*-nitrophenyl α-D-galactobiopyranoside α(1 → 2), α(1 → 3) and α(1 → 6) were isolated and characterized. α-Galactosidases with pronounced regioselectivity towards α-Gal-O-R acceptor were identified.

Keywords: α-Galactosidase; Transglycosylation; Regioselectivity; Galactobiose; Filamentous Fungi

1. Introduction

Chemical synthesis of oligoglycosides is often accompanied by a number of protection/deprotection steps. Moreover, with some thermodynamically unfavorable glycosidic linkages, as, e.g., β-Man, α-Glc and α-Gal the formation of other, unwanted, anomeric linkages is possible. Especially in these cases, the enzymatic approach is an attractive alternative. Glycosidases are more versatile than transferases in respect to the acceptor structures. However, while stereoselectivity of both types of enzymes is virtually absolute regioselectivity may be a problem with glycosidases. Regioselectivity can be partly modulated by reaction conditions, and quite

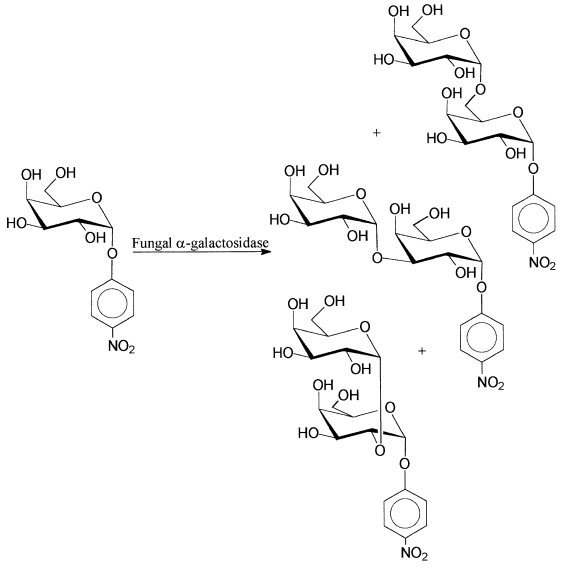

Fig. 1. Formation of *p*-NP-α-galactobiopyranosides using fungal α-galactosidases.

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strongly by the type of anomeric linkage of the acceptor (in the case of glycosidic acceptor) [1,2]. Type of OH-group in the acceptor saccharide is another important factor, primary OH group — in the hexopyranoses the 6-OH position — are more reactive. However, the decisive factor influencing the regioselectivity of respective glycosidases is their type, e.g., their source.

Previous papers described the selective formation of only α(1 → 6) [3] or α(1 → 3) galactobioses [4,5] or of their mixtures [6–8] by α-galactosidases. The α-D-(1 → 3)-linkage can be found in a number of important biological structures, e.g., *iso*-globotriose (Galα(1 → 3)Galβ(1 → 4)Glc), which is an important epitope in xenotransplantation (e.g., from pigs to humans) [9]. To our knowledge, the formation of α(1 → 2) galactobiose moiety by α-galactosidases has not been described yet.

We demonstrate here enzymatic formation of three types of *p*NP-α-galactobioses as an application of our library of fungal α-galactosidases.

2. Experimental

The strains were obtained from the Culture Collection of Fungi (CCF), Department of Botany, Char-

Table 1
Regioselectivity of fungal α-galactosidases — production of *p*NP-α-galactobioses

Source of α-galactosidase	α(1 → 6) (%)	α(1 → 2) (%)	α(1 → 3) %)[a]	Conversion[b]
Aspergillus parasiticus CCF 3058	86.1	1.7	8.6	+ + +
A. parasiticus CCF 2155	60.4	–	15.6	+
A. parasiticus CCF 1298	92.2	–	3.2	+
A. parasiticus CCF 141	80.2	–	3.0	+ +
A. flavus CCF 642	87.4	–	12.6	+ +
A. flavus CCF 146	96.6	–	3.4	+ +
A. flavus CCF 1129	93.8	–	3.9	+ +
A. tamarii CCF 3085	76.1	2.2	21.7	+ +
A. tamarii CCF 1665	91.7	–	8.3	+
A. phoenicis CCF 61	**93.6**	–	1.3	+ + +
A. oryzae CCF 1602	42.3	–	57.7	+
A. sojae CCF 3060	67.3	4.4	5.6	+ +
A. flavipes CCF 2026	41.6	**35.0**	23.4	+
A. niger CCIM K1	**90.4**	–	–	+ + +
A. terreus CCF 2539	78.8	–	–	+
A. terreus CCF 55	**100**	–	–	+ + +
A. terreus CCIM LM	93.9	–	–	+
A. terreus CCF 3059	88.7	–	8.5	+ +
A. terreus CCF 57	98.1	–	–	+ +
Talaromyces flavus CCF 2686	11.3	8.4	**80.3**	+ + +
T. flavus CCF 2324	8.0	3.5	**86.5**	+ + +
T. trachyspermus CCF 2231	95.4	2.5	–	+ + +
T. avellaneus CCF 2923	82.9	5.9	9.7	+ + +
Penicillium vinaceum *CCF 2384*	90.3	–	4.7	+
P. commune CCF 2962	93.6	–	–	+ + +
P. brasilianum CCF 2155	75.5	1.5	–	+ +
P. melinii CCF 2440	88.9	–	–	+ +
P. chrysogenum CCF 1269	88.1	7.8	–	+ +
P. daleae CCF 2365	83.7	–	5.0	+ +
Circinella muscae CCF 1568	40.9	**59.1**	–	+ + +
C. muscae CCF 2407	87.0	13.0	–	+
Green coffee beans (Sigma)	–	–	81.0	+

[a] The rest to the 100% non-identified *p*NP-glycosides.
[b] Conversion of *p*NP-α-Gal into *p*NP-α-Galactobioses (all isomers), + + + > 25%, + + > 10%, + < 10%.

les University, Prague, and from the Culture Collection of the Institute of Microbiology (CCIM), Prague, Czech Republic. The cultivation was described in our previous paper [10]. α-Galactosidase activity was assayed using *p*-nitrophenyl α-D-galactopyranoside (*p*NP-α-Gal). One unit of α-galactosidase activity was defined as the amount releasing 1 μmol of *p*-nitrophenol per minute at pH 5.0 and 35°C [10].

Screening system: *p*NP-α-Gal [11] (5 mg, 16.6 μmol) was dissolved in citrate-phosphate buffer (250 μl, 50 mM, pH 5.5) at 37°C and 1 U of the respective α-galactosidase was added. The reaction was monitored by HPLC (Waters PDA detector 996, HPLC solvent delivery system Waters 600, 250 × 4 mm column, Nucleosil 120-5 C18, mobile phase-water, flow rate 1 ml min^{-1}, detection at 300 nm).

Semipreparatory procedure was carried out using *p*NP-α-Gal (40 mg, 0.133 mmol) in citrate-phosphate buffer (50 mM, pH 5.0, 1 ml) and α-galactosidase from *Talaromyces flavus* CCM 2324 (4 U) at 37°C for 2 h. The reaction was stopped by 5-min boiling. Reaction mixture was extracted with diethyl ether (3 × 250 μl) to remove liberated *p*-nitrophenol and, after reducing volume in vacuo, loaded to Sephadex LH 20 column (2.6 × 210 cm, flow rate 24 ml h^{-1}). The column was eluted with the mixture MeOH/H_2O (4:1).

NMR: ^{1}H and ^{13}C NMR spectra were measured on a Varian INOVA-400 spectrometer (400 and 100 MHz, respectively) in D_2O at 30°C and referenced to internal TSP (δ_H 0.0) and acetone (δ_C 30.5). All experiments (HOM2DJ, gCOSY, LR COSY,

Table 2
^{1}H NMR data (400 MHz, D_2O, 30°C) of *p*NP α-galabioses (chemical shifts [ppm], coupling constant [Hz])

Proton	αGal(1 → 6) αGal-*p*NP		αGal(1 → 2) αGal-*p*NP		αGal(1 → 3) αGal-*p*NP	
J_{HH}	δ_H	J_{HH}	δ_H	J_{HH}	δ_H	J_{HH}
H-1	5.935		6.101		5.919	
$J_{1,2}$		*3.8*		*3.5*		*3.6*
H-2	4.058		4.173		4.208	
$J_{2,3}$		*10.2*		*10.3*		*10.3*
H-3	4.140		4.250		4.261	
$J_{3,4}$		*3.3*		*3.3*		*2.9*
H-4	4.102		4.112		4.323	
$J_{4,5}$		*1.1*		*1.2*		*1.0*
H-5	4.157		4.016		3.983	
$J_{5,6d}$		*8.0*		*7.4*		*7.3*
$J_{5,6u}$		*3.5*		*4.9*		*5.0*
$H\text{-}6_d$	3.823		3.707		3.707	
$H\text{-}6_u$	3.650		3.749		3.748	
$J_{6d,6u}$		*10.6*		*11.9*		*11.9*
H-1′	4.820		5.140		5.229	
$J_{1',2'}$		*3.8*		*4.0*		*3.9*
H-2′	3.680		3.797		3.911	
$J_{2',3'}$		*10.2*		*10.3*		*10.3*
H-3′	3.306		3.962		3.996	
$J_{3',4'}$		*3.4*		*3.3*		*3.3*
H-4′	3.868		4.020		4.055	
$J_{4',5'}$		*1.2*		*1.3*		*1.3*
H-5′	3.851		4.202		4.254	
$J_{5,6'd}$		*7.3*		6.9		n.d.[a]
$J_{5',6'u}$		*2.5*		*6.9*		n.d.
$H\text{-}6'_d$	3.760		3.773		3.799	
$H\text{-}6'_u$	n.d		–		n.d.	

[a] n.d. not determined. Signals of *p*NP moiety: Galα(1 → 6) Galα-*p*NP — 7.337 and 8.298 (4 H, AA'BB', $\sum J = 9.3$ Hz); Galα(1 → 2) Galα-*p*NP — 7.354 and 8.287 (4 H, AA'BB'. $\sum J = 9.3$ Hz); Galα(1 → 3) Galα-*p*NP–7.318 and 8.284 (4 H, AA'BB', $\sum J = 9.3$ Hz).

TOCSY, HMQC, HMBC, NOE difference, 1D-TOCSY) were performed using either standard Varian pulse sequences or those obtained from Varian User Library.

3. Results and discussion

Thirty-one extracellular α-D-galactosidases of fungal origin (*Penicillium brasilianum*, *P. vinaceum*, *P. commune*, *P. melinii*, *P. daleae*, *Circinella muscae* (two strains), *Talaromyces flavus* (two strains), *T. trachyspermus*, *T. avellaneus*, *Aspergillus tamarii* (two strains), *A. sojae*, *A. phoenicis*, *A. flavofurcatis*, *A. parasiticus* (four strains), *A. flavipes*, *A. flavus* (three strains), *A oryzae*, *A niger*, *A. terreus* (five strains)) were prepared [10] and screened for the regioselectivity in transglycosylation of *p*NP-α-Gal (Fig. 1, Table 1). With some enzymes, more regioisomers were formed and the α(1 → 6) isomer prevailed. α-Galactosidase from *T. flavus*, *A. flavipes* and *C. muscae* produced high proportion of the α(1 → 2) isomer. Some enzymes displayed selective preference to α(1 → 6) isomer, e.g., α-galactosidase from *A. phoenicis*, *A. niger* and *A. terreus*. Linkage α(1 → 3) was formed in high yield by α-galactosidase from *T. flavus*. Commercial α-galactosidase from green coffee beans produced this linkage as well, however, in considerably lower yield.

Standards for HPLC were obtained from the reaction mixture catalyzed by α-galactosidase from *T. flavus* 2324. Pure *p*-nitrophenyl α-galactobioses: α(1 → 2) 1.6 mg, 5.2%; α(1 → 3) 5.7 mg, 18.5%; α(1 → 6) 0.7 mg, 2% were isolated using gel chromatography, in spite of the identical molecular mass. This separation was possible probably because of the different exclusion volumes of the respective regioisomers. Their NMR signals were assigned (Tables 2 and 3) by HOM2DJ, COSY, TOCSY, HMQC, and HMBC experiments. The first galactose unit was identified on the basis of NOE between H-1 and *ortho*-*p*NP protons and heteronuclear coupling between H-1 and C_{ipso} of *p*NP. Reported proton-proton couplings were extracted using ^{1}H NMR, HOM2DJ, and 1D-TOCSY. The linkage position was determined from the characteristic downfield shifts of the involved carbon (bold entries in Table 3) and supported by heteronuclear couplings across

Table 3
^{13}C NMR data (100 MHz, D_2O, 30°C) of *p*NP α-galabioses (chemical shifts, ppm)

Carbon	αGal(1 → 6) αGal-*p*NP	αGal(1 → 2) αGal-*p*NP	αGal(1 → 3) αGal-*p*NP
C-1	97.8	94.5	97.1
C-2	67.9	**72.1**[e]	66.6
C-3	69.7	68.1	**74.5**[e]
C-4	69.5	69.3	65.7
C-5	70.8	72.3	72.3
C-6	**66.9**[e]	61.2	61.2
C-1′	96.6	96.2	95.5
C-2′	68.2	68.2	68.5
C-3′	69.6	69.3	69.6
C-4′	69.3	69.4	69.6
C-5′	70.9	71.3	71.4
C-6′	61.1	61.0	61.5
C-*ipso*	n.d.[d]	161.7[b]	161.7
C-*ortho*	117.4	117.3	117.1
C-*meta*	126.2	126.2	126.3
C-*para*	n.d.	142.6[b]	142.7

[a] From HMQC.
[b] From HMBC.
[c] Direct observation.
[d] n.d. not determined.
[e] Site of the attachment of the second α-Gal.

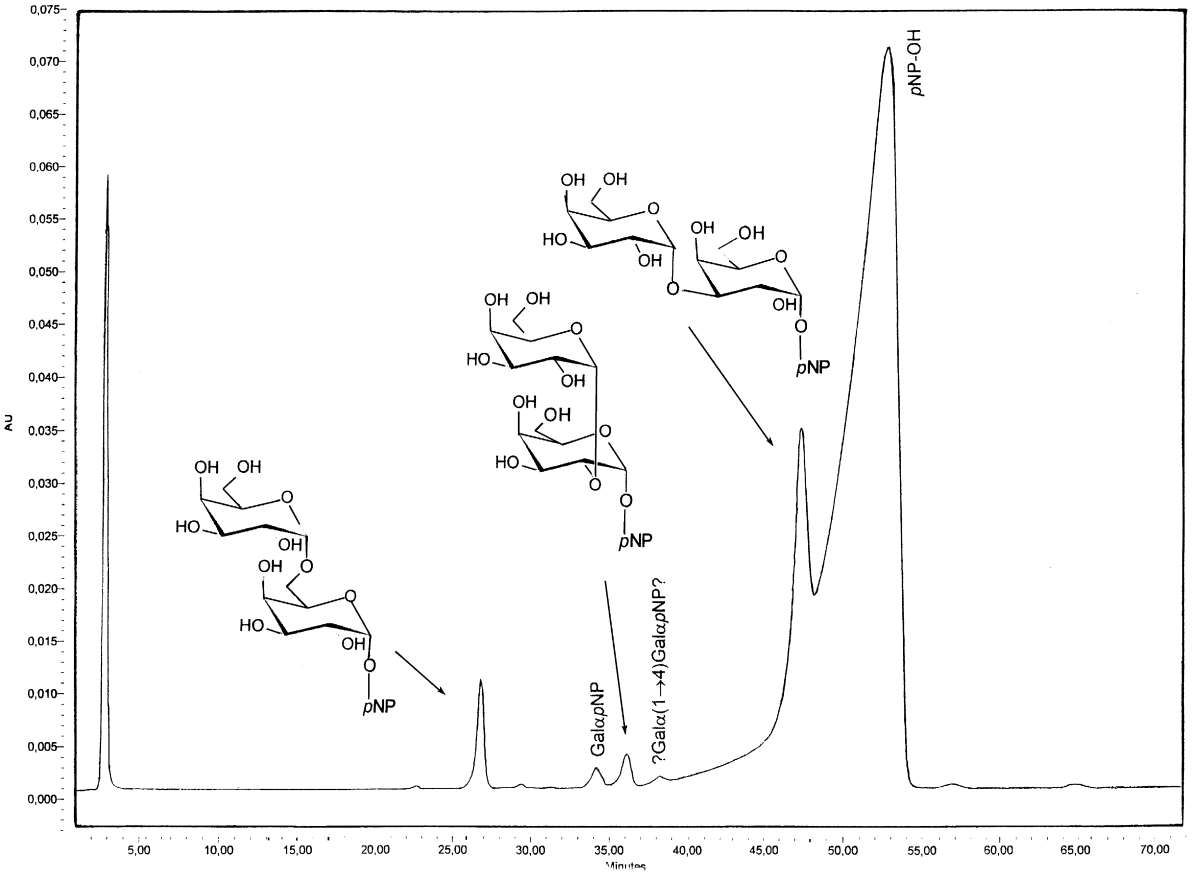

Fig. 2. Transglycosylation of *p*NP-α-Gal catalyzed by *Talaromyces flavus* CCF 2324, HPLC, 250 × 4 mm column, Nucleosil 120-5 C18, mobile phase-water, flow rate 1 ml min^{-1}, detection 300 nm.

the glycosidic bond (H-3 to C-1′ and H-1′ to C-3, H-2 to C-1′ and H-1′ to C-2) observed by HMBC.

Besides the identified products, another minor peak was observed in HPLC. The peak was located between isomers $\alpha(1 \rightarrow 2)$ and $\alpha(1 \rightarrow 3)$ (Fig. 2) and its UV spectrum was identical to that of other *p*NP-galabioses. From the r.t. and spectral characteristic, we assume that some of these unknown peaks could be ascribed to $\alpha(1 \rightarrow 4)$ isomer. Due to paucity of the compound, its identification could not be performed; nevertheless, its isolation in larger amount is under progress.

Our results clearly depict α-galactosidases with high regioselectivity combined with high transglycosylation yields. These findings are especially valuable for further exploitation of our enzyme library for targeted α-galactosylation of other glycosidic substrates.

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