Carbohydrate Research 346 (2011) 602–605

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Activity of *Debaryomyces hansenii* UFV-1 α-galactosidases against α-D-galactopyranoside derivatives

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ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 10 January 2011
Accepted 20 January 2011
Available online 28 January 2011

Keywords:
α-Galactosidases
Debaryomyces hansenii UFV-1
α-D-Galactopyranoside derivatives
Inhibition

ABSTRACT

α-D-Galactopyranosides were synthesized and their inhibitory activities toward the *Debaryomyces hansenii* UFV-1 extracellular and intracellular α-galactosidases were evaluated. Methyl α-D-galactopyranoside was the most potent inhibitor compared to the others tested, with K_i' values of 0.82 and 1.12 mmol L^{-1}, for extracellular and intracellular enzymes, respectively. These results indicate that the presence of a hydroxyl group in the C-6 position of α-D-galactopyranoside derivatives is important for the recognition by *D. hansenii* UFV-1 α-galactosidases.

1. Introduction

Numerous glycosidases have been isolated and fully characterized, and they have been classified into families and groups according to their amino acid sequences. Enzymes of the same group usually have common catalytic mechanisms.[1] The anomeric specificity of the glycosidases is usually rigid, but the regioselective specificity for the other glycosidic positions is more variable among the glycosidases. Several studies have reported that hydrolytic reactions with analogous deoxy derivatives of the appropriate glycon can be catalyzed by D-glycosidases, but the tolerance of the active site to the position of deoxygenation varies between enzymes from different sources.[2–5] Despite the widespread use of iminosugars as transition-state analogous inhibitors of glycosidases, inhibitors based on modifications of conventional carbohydrates are still valuable.[6]

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) have gained considerable attention as industrial catalysts due to their potential biotechnological applications since they can be isolated from organisms, such as yeasts that are generally recognized as safe (GRAS).[7,8] These glycosyl hydrolases are widely distributed in microorganisms, plants, and animals.[9–12] α-D-Galactoside galactohydrolase catalyzes the hydrolysis of the terminal nonreducing α-galactosyl residues from various α-galactosides.[13] The production of intracellular and extracellular α-galactosidases from *Debaryomyces hansenii* UFV-1 cultivated in a medium containing galactose as the carbon source was recently reported.[14] The substrate specificities of these enzymes were investigated by using synthetic substrates, galactose-containing oligosaccharides, and polymers. *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases were highly selective, showing absolute specificity for the α-galactosyl bond. These enzymes hydrolyzed *p*NPαGal but showed no activity for other synthetic substrates, such as glucosidic derivatives or for β-linked glycosides. D-Galactose inhibited the intracellular enzyme uncompetitively, but was shown to be a noncompetitive inhibitor for the extracellular α-galactosidase.[14,15] Since, galactose was an inhibitor for both *D. hansenii* UFV-1 α-galactosidase isoforms,[14,15] we decided to synthesize some of its derivatives to search for new possible lead compounds as reversible inhibitors of the extracellular and intracellular α-galactosidases.

The aim of the present study was to evaluate the influence of substituents in the pyranosidic ring of some α-D-galactopyranoside derivatives, coupled to some variation in the aglycon moiety on the hydrolytic activity of extracellular and intracellular *D. hansenii* UFV-1 α-galactosidase isoforms. Structure and inhibitory activity relationships concerning these α-D-galactopyranoside derivatives, as well as 4-nitrophenyl α-D-galactopyranoside derivatives are discussed here.

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doi:10.1016/j.carres.2011.01.024

2. Experimental

2.1. General methods

Solvents were dried and distilled before use. All reactions were monitored by TLC on Silica Gel 60 (0.25 mm) followed by spraying the plates with 15% ethanolic H_2SO_4 and heating at ca. 200 °C. Column chromatography was performed on silica gel (0.035–0.070 mm). ^1H and ^{13}C NMR spectra were recorded with a Bruker DPX200 instrument at 200 and 50 MHz, respectively. High-resolution electrospray-ionization mass spectra were recorded on a MicroTOF Bruker instrument of the Mass Spectrometry Service of the University of São Paulo.

The *p*NPαGal (4-nitrophenyl α-D-galactopyranoside) was obtained from Sigma–Aldrich Chemical Co. Methyl α-D-galactopyranoside (**1**) was purchased from Sigma–Aldrich. Methyl 6-*O*-(4-toluenesulfonyloxy)-α-D-galactopyranoside (**2**), methyl 6-azido-6-deoxy-α-D-galactopyranoside (**3**) and 4-(acetylamino)phenyl α-D-galactopyranoside (**4**) were prepared according to described procedures.[16–18]

2.2. 4-Nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside (5)

To a solution of 4-nitrophenyl 6-*O*-tosyl-α-D-galactopyranoside[19] (0.12 g, 0.26 mmol) in 5 mL of dry pyridine, 2 mL of Ac_2O was added dropwise. The reaction mixture was kept at 0 °C for 18 h. The mixture was poured onto ice and concd HCl solution was added until pH 1. The resulting aqueous solution was extracted with EtOAc (3 × 30 mL). The combined organic phase was then washed with satd aq Na_2CO_3 and then with distilled water, dried with anhyd Na_2SO_4, and after filtration, concentrated to dryness in a rotatory evaporator. The crude peracetate was dissolved in 3 mL of dry DMF and sodium azide (0.14 g, 2.15 mmol) was added. The reaction mixture was heated at 65 °C for 72 h and then the solvent was removed by evaporation. The crude product was dissolved in a two-phase system of 20 mL of cold water and 20 mL of EtOAc. The resulting solution was transferred to a separatory funnel, and after shaking, the organic layer was collected. The aqueous phase was further extracted with EtOAc (2 × 20 mL), the combined organic phase was treated with dry Na_2SO_4, filtered, and concentrated in a rotatory evaporator. The residue was stirred overnight at room temperature with a solution of NaOMe prepared by adding a catalytic amount of sodium to 10 mL of dry MeOH.[20] The solution was neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated to dryness, and the residue was submitted to column chromatography (9:1 EtOAc–hexane) to furnish the desired compound (0.056 g, 65% overall yield) as a light-yellow solid: mp 170 °C (decomp.), $[\alpha]_D^{21}$ +125 (*c* 0.4, MeOH).

^1H NMR (CD_3OD, 200 MHz): δ 8.22 (d, 2H, J = 9.2 Hz), 7.32 (d, 2H, J = 9.2 Hz), 5.74 (d, 1H, J = 2.2 Hz), 4.10–3.88 (m, 3H, H-2, H-3, H-4), 3.68–3.49 (m, 2H, H-5, H-6a), 3.23 (dd, 1H, J 6a,6b = 12 Hz, J 6b,5 = 3.7 Hz, 6b). ^{13}C NMR (CD_3OD, 50 MHz): δ 163.4 (C-1′), 143.8 (C-4′), 126.6 (C-3′), 117.8 (C-2′), 99.1 (C-1), 72.9, 71.1, 70.9, 69.3 (C-2, C-3, C-4, C-5, interchangeables), 52.5 (C-6). HRESIMS calcd for $C_{12}H_{14}N_4O_7Na$: m/z 349.0762; found: m/z 349.0764 [M+Na^+].

2.3. α-Galactosidase assay

D. hansenii UFV-1 α-galactosidases purified by two-step chromatography as previously described[14,15] were used in this study. The hydrolytic activities of the α-galactosidases were determined by measuring the release of 4-nitrophenol (*p*NP) from 4-nitrophenyl α-D-galactopyranoside (*p*NPαGal). The α-galactosidase assay contained 650 μL of 0.1 mol L^{-1} NaOAc buffer, pH 5.0, 100 μL of enzyme solution (1.02 μg protein mL^{-1}), and 250 μL of 2 mmol L^{-1} *p*NPαGal. The mixture was incubated at 60 and 55 °C for the extracellular and intracellular α-galactosidases, respectively. The amount of *p*NP released was determined at 25 °C at 410 nm and calculated according to a standard curve (0–0.16 μmol *p*NP).

One enzyme unit (U) was defined as the amount of enzyme that released 1.0 μmol of product per minute under the assay conditions.

2.4. Inhibitory activity determination

The inhibition constants (K_i or K_i') of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases for each galactoside derivative were calculated from the Dixon plot. The *p*NPαGal concentrations were 0.4, 0.8, 1.2, 1.6, and 2.0 mmol L^{-1} and the concentrations of the inhibitors (galactoside derivatives) were 0.5, 1.0 and 2.0 mmol L^{-1}.

2.5. Determination of protein concentration

Protein concentration in the enzymatic preparations was determined by the Coomassie Blue binding method (Bio-Rad Protein Assay) with bovine serum albumin (BSA) as the standard.[21]

3. Results and discussion

Based on the results presented in Table 1, modifications on the galactopyranoside ring had a significant influence in the interaction of these compounds with the *D. hansenii* UFV-1 α-galactosidases. None of the derivatives analyzed (Fig. 1) were substrates for the enzymes; all were inhibitors.

Methyl α-D-galactopyranoside (**1**) was the most potent inhibitor of the compounds studied (Table 1 and Fig. 2). The extracellular and intracellular α-galactosidases were uncompetitively inhibited by this compound, with K_i' values of 0.82 and 1.12 mmol L^{-1}, respectively. Compound **1** inhibited the α-intracellular galactosidase isoform with an affinity comparable to that of D-galactose (Fig. 1 and Table 1). The monosaccharide D-galactose, a final product of the reactions catalyzed by α-galactosidases, noncompetitively inhibited the extracellular α-galactosidase (K_i = 2.7 mmol L^{-1})[14] and uncompetitively (K_i' = 0.7 mmol L^{-1}) the intracellular enzyme.[15] Comparing the data for methyl α-D-galactopyranoside with those obtained for D-galactose suggests that the hydrogen bond donor capability of the anomeric center can be removed with no deleterious consequence for the inhibitory effect. Nevertheless, this modification affects the binding mode for the extracellular isoform, since galactose is a noncompetitive inhibitor while methyl α-D-galactopyranoside is an uncompetitive one.

Methyl 6-*O*-(4-toluenesulfonyl)-α-D-galactopyranoside (**2**), and methyl 6-azido-6-deoxy-α-D-galactopyranoside (**3**), were synthesized for comparison with the parent methyl galactoside derivative (**1**). The derivatives of compound **1** present groups of different chemical nature in the C-6 position (a bulky lipophilic group and a smaller hydrophilic group, respectively) (Fig. 1). Thus, the importance of the hydroxyl group in the C-6 position, as well as the effect of the groups with distinct properties could be evaluated concerning the recognition of the enzymatic active sites. A significant reduction of inhibitory effect occurred for compounds **2** and **3** in comparison with that of compound **1**, which indicated that the hydroxyl group in C-6 is important for the recognition of the active sites of *D. hansenii* UFV-1 α-galactosidases (Table 1). There are a few studies in literature concerning inhibition of α-galactosidase

Table 1
Inhibitory activity (K_i or K'_i) of α-D-galactopyranoside derivatives against *Debaryomyces hansenii* UFV-1 α-galactosidases

Compound	Inhibition		K_i or K'_i (mmol L^{-1})	
	Extracellular	Intracellular	Extracellular	Intracellular
1	Uncompetitive	Uncompetitive	0.82 ± 0.01 (K'_i)	1.12 ± 0.03 (K'_i)
2	Competitive	Competitive	6.00 ± 0.02	2.33 ± 0.02
3	Uncompetitive	Competitive	9.35 ± 0.02 (K'_i)	2.45 ± 0.01
4	Competitive	Noncompetitive	3.03 ± 0.03	5.53 ± 0.03
5	ND[a]	ND[a]	>20	>20
Galactose	Noncompetitive	Uncompetitive	2.7 ± 0.01	0.7 ± 0.02 (K'_i)

[a] ND: not determined under assay conditions used.

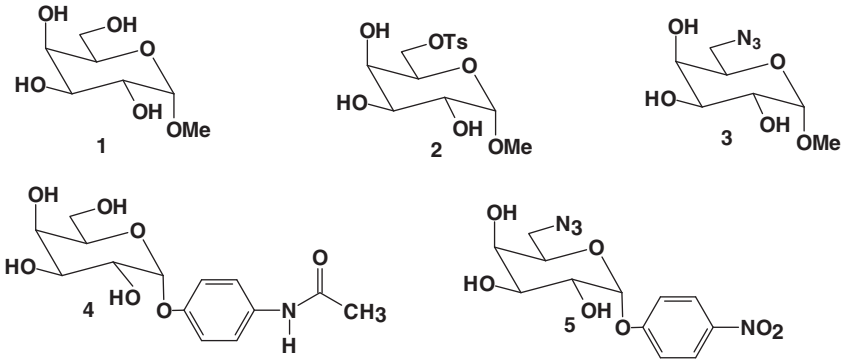

Figure 1. α-D-Galactopyranoside derivatives: Methyl α-D-galactopyranoside (**1**); methyl 6-*O*-(4-toluenesulfonyl)-α-D-galactopyranoside (**2**); methyl 6-azido-6-deoxy-α-D-galactopyranoside (**3**); 4-(acetylamino)phenyl α-D-galactopyranoside (**4**); 4-nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside (**5**).

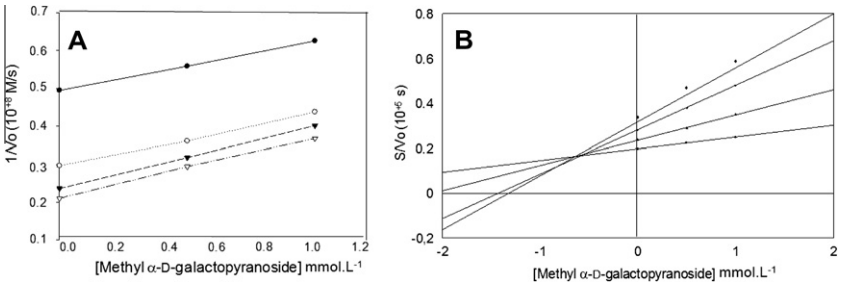

Figure 2. Inhibition of *Debaryomyces hansenii* UFV-1 extracellular α-galactosidase by methyl α-D-galactopyranoside at different concentrations (0, 0.5 and 1.0 mmol L^{-1}). (A) Dixon plots in different substrate concentrations (mmol L^{-1}): 0.4 (●); 0.8 (○); 1.2 (▲) and 1.6 (△); (B) Cornish–Bowden plots in different substrate concentrations (mmol L^{-1}): 0.4 (●); 0.8 (○); 1.2 (▲) and 1.6 (△).

activity using synthetic compounds. The presence of three hydroxyl groups (at the C-3, C-4, and C-6 positions) in the *p*NPαGal substrate is necessary for its derivatives to be substrates of *Aspergillus niger* α-galactosidase, but coffee bean α-galactosidase tolerates both the 2- and 6-deoxy derivatives.[2] Compound **2** was a slightly more potent inhibitor than compound **3**, especially for the extracellular α-galactosidase isoform (Fig. 1 and Table 1), but a direct comparison must be viewed with care since the inhibition mode is different for the two compounds. This difference could be attributed to the more lipophilic character of compound **2** and more efficient desolvation when this galactoside derivative is transferred to the enzymatic binding site. Thus, synthesis of glycosyl derivatives with hydrophobic substituents at the anomeric carbon could be a good strategy to search for more potent inhibitors. Other modifications in the C-6 position are necessary to furnish information about the possibility of substituting hydrophobic moieties for the C-6 hydroxyl group.

The compounds 4-(acetylamino)phenyl α-D-galactopyranoside (**4**) and 4-nitrophenyl 6-azido-6-deoxy-α-D-galactopyranoside (**5**) were prepared to be initially assayed as substrates or inhibitors of the glycosyl hydrolases isoforms (Fig. 1). The aromatic galactoside **4** is a derivative of 4-nitrophenyl α-D-galactopyranoside in which the nitro group was reduced and acetylated with the aim of evaluating the capability of the α-galactosidases to hydrolyze aryl α-D-galactopyranosides with electron-releasing groups instead of electron-withdrawing ones. Finally, compound **5** was prepared to evaluate whether the presence of the azido group at C-6 influences the α-galactosidase activity in comparison with the parent substrate 4-nitrophenyl α-D-glucopyranoside. Preliminary experiments carried out with a partially purified β-glycosidase from *Biomphalaria glabrata* (unpublished data) showed that identical substitution in 4-nitrophenyl β-D-glucopyranoside led to the corresponding 6-azido derivative that was an enzymatic inhibitor with a K_i in the range of 1.0 mmol L^{-1}.

In the series of the aromatic galactosides, it was observed that a variation of the substituent groups promoted significant influence on the behavior of those derivatives when compared with *p*NPα-Gal. Galactoside **4** was an inhibitor, though less potent than **1**, confirming the previous suggestion (Fig. 1 and Table 1). Galactoside **4** is an inhibitor while *p*NPαGal is a substrate, probably because the

aglycon of **4** is a poorer leaving group (4-acetylaminophenol) than that of *p*NPαGal (4-nitrophenol) due to its electron-releasing character. However, it is important to consider that the binding of the *N*-acetyl group to the enzymatic active site may also contribute to its recognition as an inhibitor.

Compound **5**, which presents substitution of an azido group for the C-6 hydroxyl group of *p*NPαGal, did not show the ability to bind to the α-galactosidases. Considering that it was not possible to determine the K_i values of both α-galactosidase isoforms for compound **5** under the assay conditions, it seems plausible to assume that this substance is not recognized by the enzymes, evidencing once more the poor binding of azido group in the C-6 position already observed for derivative **3**.

It is interesting to note that different inhibition types for the extracellular and intracellular isoforms are observed for compounds **3** and **4** (Table 1). This observation is relevant as an additional evidence for the presence of distinctive isoforms, with identical N-terminal sequences and molecular masses but having a different amino acid composition and glycosylation pattern.[14,15] Furthermore, spectroscopic and thermodynamic properties have recently been determined for *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases, suggesting that these enzymes have different behaviors although they possess some similar secondary structures.[22]

For us, the data suggest a second site of interaction for some of α-D-galactopyranoside derivatives, in addition to the active site. It is also promising that the α-D-galactopyranoside derivatives investigated here can be lead compounds to develop specific inhibitors for each α-galactosidase isoform through further chemical modification.

Acknowledgments

This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais—FAPEMIG, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES, and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq, Brazil.

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