Improvements in Enzymatic Preparation of Alkyl Glycosides

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Abstract

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Three glycosides of middle chain aliphatic alcohols (hexanol and heptanol) were prepared by enzymatic glycosidation. The preparation of hexyl β-D-glucopyranoside was achieved in moderate yields via reverse hydrolysis catalysed by defatted meals from almond, apricot, and peach kernels. The apricot meal as a cheap source of β-glucosidase was found to have catalytic efficiency resembling to that of the almond meal. Hexyl and heptyl β-D-galactopyranosides were prepared from D-lactose by transgalactosidation catalysed by commercial β-galactosidases from *Aspergillus oryzae* in two-phase system. The improvement of the product yields was achieved by a simple replacement of the organic phase (serving as a pool of acceptor alcohol and extracted product) with a fresh portion of the acceptor.

Keywords: alkyl glycosides; plant seed glycosidases; β-glucosidase; β-galactosidase

Alkyl glycosides bearing long aliphatic aglycones belong to the easily degradable nonionic surfactants used in cosmetics, biochemistry, and pharmaceutical industry (Amalric & Lecocu-Michel 1997; Garcia *et al*. 1997; Meezan & Pillion 1997). Their chemical production involves several synthetic steps and often leads to mixtures of both anomers of two possible cyclic forms of the glycoside. This results in lower chemical yields of the target substance and in its complicated purification. Simple, cheap, and effective enzymatic approaches are therefore highly desirable. For such syntheses, glycosidases are used with advantage in a far cheaper and simplier way as compared to another group of glycosylating enzymes – glycosyl transferases.

Generally, two methods may be employed in one-step enzymatic preparation of alkyl glycosides – transglycosidation and reverse hydrolysis. Reverse hydrolysis is a thermodynamic process leading to an equilibrium between the target glycoside and starting monosaccharide while transglycosidation employs partial transferase activity of glycosidases. Glycosidase thus catalyses the transfer of monosaccharide from the nonreducing end of the substrate (polysaccharide, oligosaccharide, or glycoside) to a nucleophil (an alcohol or another molecule of the saccharide) present in the reaction mixture (van Rantwijk *et al*. 1999). The level of transferase activity may strongly differ case to case with every glycosidase while monosaccharides are

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formed as hydrolytic side-products. The development of techniques increasing the product yield as well as the search for new sources of enzymes is therefore still topical.

Agricultural materials may serve as sources of various enzyme activities with an industrial potential. Besides the notoriously studied β-glucosidase from almond kernel, the applications of apple (Tong *et al.* 2004; Lu *et al.* 2007; Yu *et al.* 2007, 2008), apricot, peach, and loquat (Tong *et al.* 2004) seeds were tested in preparative glycosidations.

The purpose of this study was to find ways to improve the economy of glycosidase-catalysed production of alkyl glycosides from D-glucose, D-galactose, and D-lactose either by means of the use of cheap alternatives to commercial enzymes or via an improvement of the reaction conditions.

MATERIAL AND METHODS

Material. Lactase F (*Aspergillus oryzae*) was a gift from Amano (Nagoya, Japan), Fungal lactase (*Aspergillus oryzae*) was obtained from Shin Nihon (Aichi, Japan), *o*-nitrophenyl β-D-galactopyranoside was purchased from Sigma, Chemical Co. (St. Louis, USA), *p*-nitrophenyl α-D-galactopyranoside and *p*-nitrophenyl β-D-glucopyranoside came from Acros Organics (Geel, Belgium), *p*-nitrophenyl β-D-xylopyranoside from Institute of Chemistry SAS (Bratislava, Slovakia). Almond kernels, Brazil nuts, pumpkin seeds, and lentils were purchased on the local market. Peach and apricot kernels came from the author's garden. All other chemicals were from domestic distributors.

Preparation of plant glycosidase solutions, After the breaking of the stone shells, kernels were soaked in water and their outer skin was peeled manually. The peeled kernels were blended on a knife homogeniser, dried in air at laboratory temperature, and extracted with diethyl ether in Soxhlet apparatus to remove fats. Dry extracted powder was dispersed in 0.05M acetate buffer pH 5 (1 g/10 ml), stirred for 1 h and filtered. The filtrate was used for the assay of the enzymatic activity, protein content, and for preparative reactions.

Protein assay. Protein determination was performed using the method by Bradford (1976). Bovine serum albumin was used as the standard.

Glycosidase assay. Hydrolysis of the appropriate nitrophenyl glycosides to the corresponding nitrophenol was used to determine the activities of glycosidases in the kernel extracts. An amount of 100 µl of the extract was mixed with 750 µl of 5mM solution of the respective nitrophenyl glycoside in 0.05M acetate buffer, pH 5. The enzymatic reaction was terminated by the addition of 1.5 ml of 1M Na_2CO_3. The formation of *p*- or *o*-nitrophenol was measured by a spectrophotometer at 410 nm and the respective amount was calculated from the calibration curve. One glycosidase unit (U) is defined as the amount of enzyme that releases 1 µmol of the respective nitrophenol per minute.

Preparation of n-hexyl β-D-glucopyranoside. D-Glucose (7 g) was dissolved in 30 ml of the meal extract and stirred with 180 ml of hexanol for 7 days at laboratory temperature. The two-phase mixture was separated in a separation funel. Alcohol fraction was heated in boiling water bath for 20 min, filtered and extracted with water to remove traces of sugar, salts, and enzyme, and was subsequently evaporated to dryness using repeated additions of water to remove the remaining alcohol. The product was recrystallised from methanol.

NMR spectra of n-hexyl β-D-glucopyranoside:

^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 0.87 (t, 3H, CH_3), 1.27–1.35 (m, 6H, 3 × CH_2), 1.53–1.64 (m, 2H, CH_2), 3.29 (dd, 1H, $J_{2,3}$ = 8.40 Hz, $J_{3,4}$ = 8.60 Hz, H-3), 3.12 (dd, 1H, $J_{1,2}$ = 7.80 Hz, H-2), 3.50–3.54 (m, 2H, H-5, 1 × OCH_2), 3.57–3.58 (m, 1H, H-4), 3.82–3.87 (m, 3H, 1 × OCH_2, H-6a, H-6b), 4.30 (d, 1H, H-1).

^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 14.1 (C-6'), 22.7, 25.5, 31.7, 32.6 (C-2', C-3', C-4', C-5'), 61.3 (C-6), 69.4 (C-4), 70.5 (C-1'), 73.3, 75.6, 76.4 (C-2, C-3, C-5), 102.8 (C-1).

Transgalactosidation. 80 ml of the respective alcohol were added to the solution of 0.8M D-lactose in 0.05M acetate buffer pH 5 (20 ml) followed by 70 mg (6.6 kU) of Fungal lactase or 220 mg (4 kU) of Lactase F. After the predefined time of stirring at laboratory temperature, the two phases were separated in a separation funel. The isolation of the products was performed in the same way as given above. The products were recrystallised from ethanol. In two-step reaction, the remaining water phase was overlaid with another portion (50 ml) of the respective alcohol and stirred for another time period. The alcohol fractions after both reaction steps were collected and processed in the obvious way.

NMR spectra of n-hexyl β-D-galactopyranoside:

^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 0.86 (t, 3H, CH_3), 1.20–1.35 (m, 6H, 3 × CH_2), 1.48 (m, 2H, OCH_2-2'), 3.22–3.33 (m, 3H, H-2, H-3, H-5), 3.39

(dt, 1H, $J_{5,6a}$ = 6.9 Hz, $J_{6a,6b}$ = 9.6 Hz, H-6a), 3.44–3.56 (m, 2H, CH_2-1'), 3.62 (m, 1H, H-4), 3.72 (dt, 1H, $J_{5,6b}$ = 6.6 Hz, H-6b), 4.04 (dt, 1H, $J_{1,2}$ = 7.5 Hz, $J_{1,CH2\text{-}1'}$ = 3.8 Hz, H-1), 4.32 (d, 1H, OH), 4.54 (t, 1H, OH), 4.67 (d, 1H, OH), 4.78 (d, 1H, OH).

^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 14.0 (C-6'), 22.1, 25.2, 29.3, 31.2 (C-2', C-3', C-4', C-5'), 60.4 (C-1'), 68.1 (C-4), 68.5 (C-6), 70.6, 73.5, 75.1 (C-2, C-3, C-5), 103.5 (C-1).

NMR spectra of n-heptyl β-D-galactopyranoside:

^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 0.86 (t, 3H, CH_3), 1.22–1.33 (m, 8H, 4 × CH_2), 1.51(q, 2H, CH_2-2'), 3.23–3.27 (m, 2H, H-2, H-3), 3.28 (bt, 1H, H-5), 3.49 (ddd, *J* = 11.1, 5.7, 5.1 Hz, 2H, H-6a, H-6b), 3.55 (ddt, 2H, CH_2-1'), 3.59–3.63 (m, 1H, H-4), 4.04 (d, *J* = 7.5 Hz, 1H, H-1), 4.31 (bd, *J* = 4.5 Hz, 1H, OH), 4.54 (bt, 1H, OH), 4.65 (bs, 1H, OH), 4.77 (bs, 1H, OH).

^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 13.9 (C-7'), 22.1, 25.5, 28.6, 29.3, 31.3 (C-2', C-3', C-4', C-5', C-6'), 60.4 (C-6), 68.2 (C-4), 68.4 (C-1'), 70.5, 73.5, 75.1 (C-2, C-3, C-5), 103.44 (C-1).

Reverse hydrolysis catalysed by β-galactosidases. 120 ml of hexanol were added to the solution of 1M D-galactose in 0.05M acetate buffer pH 5 (20 ml) followed by 70 mg (6.6 kU) of Fungal lactase or 220 mg (4 kU) of Lactase F. The reaction mixture was stirred for 5 days at laboratory temperature and then boiled under reflux for 20 min to terminate the reaction. After the phase separation, the organic phase was filtered, extracted with 18 ml of water and treated in the same way as previously. The spectra of the product were identical with the spectra of *n*-hexyl β-D-galactopyranoside described above.

RESULTS AND DISCUSSION

The screening of glycosidases in seed meals showed interesting levels of β-glucosidase in almonds, apricot, and peach kernels (Table 1). Contrary to the results of Lu *et al.* (2007), the activity of this enzyme was one order of magnitude higher, the highest level having been found in almond kernels. The differences between our results and those obtained in the cited paper may have come from different pH values of assay solutions (pH 7 in the cited report). All other seed materials tested revealed activities that were either negligible or accompanied with the enzyme activity specific to the opposite anomer.

The suitability of the three types of kernels (almond, apricot, and peach) in synthetic reactions was tested in the preparation of *n*-hexyl β-D-glucopyranoside. The price of D-cellobiose as D-glucose donor is too high to make the tranglucosidation profitable, therefore only reverse hydrolysis starting from free D-glucose was executed (Figure 1).

A preliminary experiment using the meal from almond kernel as catalyst in glucosidation of *n*-pentanol gave 12%^{mol} of the product – *n*-pentyl β-D-glucopyranoside (details not shown). The product isolation was however complicated by formation of emulsion on the phase interface. Only filtered extracts were therefore used in further glucosidations. Compared to the reaction with pentanol, the yields of *n*-hexyl β-D-glucopyranoside were significantly lower, reaching only 5.0%, 4.7%, and 2.8% for the extracts from almond, apricot, and peach kernel meals, respectively. The product was very simply isolated by evaporation of the washed hexanol phase. Its structure was identi-

Table 1. Content of proteins (mg per g of dry powder) and glycosidase activities (U per g of dry powder) in plant isolates and commercial glycosidases

	Proteins (mg/g)	α-Galactosidase	β-Galactosidase	β-Glucosidase	B-Xylosidase
		(U/g)			
Almond	20.7	2.2	46.5	323.6	0.03
Apricot	16.1	0.8	24.8	181.2	2.1
Peach	19.9	0.6	19.0	110.3	1.9
Pumpkin	12.1	0.6	11.9	0.6	0.03
Chestnut	8.7	0.5	0.6	0.0	0.0
Brazil nut	17.5	traces	0.3	0.0	0.0
Lentil	9.3	0.1	0.4	0.0	0.0
Lactase F	492.8	36.0	18.1×10^3	20.1	2.0
Fungal lactase	579.7	19.2	95.7×10^3	32.4	33.3

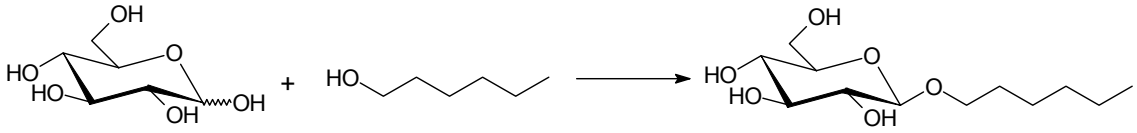

Figure 1. Synthesis of *n*-hexyl β-D-glucopyranoside by reverse hydrolysis catalysed by kernel meals

fied by NMR analysis, which confirmed that no α-anomer had been formed. The lower yields, besides a slightly lower reactivity of *n*-hexanol, may be attributed especially to the fact that only part of glucosidase was probably extracted into the buffer, its real activity in ground kernel being higher. Moreover, to simplify the isolation technique and avoid column chromatography, we gave up that portion of the product which was not extracted into the hexanol phase. Our experiments anyway proved that especially the apricot kernel meal is a promising cheap alternative to almond β-glucosidase. This fact is in agreement with the report of Lu *et al.* (2007).

Since kernel meals contained rather low activities of β-galactosidase, the synthesis of *n*-alkyl β-D-galactopyranosides was executed using two preparations of β-galactosidases from *Aspergillus oryzae*. Reverse hydrolysis catalysed by Fungal lactase and Lactase F gave, respectively, 6.3% and 5.7% of *n*-hexyl β-D-galactopyranoside relative to the starting amount of D-galactose.

The preparation of alkyl β-D-galactopyranosides, however, frequently employs the strategy of transglycosidation since D-lactose as the D-galactosyl donor is considerably cheaper than D-galactose (Figure 2). Transgalactosidation gives higher product yields in a shorter time and the formation of the opposite anomer is minimised. Increasing the chemical yield is anyway still an actual task in transglycosidation, especially when longer aliphatic alcohols are to be glycosylated. The optimisation of the reaction usually focuses on the choice of the catalyst and the relative ratio of reactants. Special techniques as the cosolvent use or modification of glycosidase with surfactants have also been studied (Okahata & Mori 1998; Lu *et al.* 2007).

Since transglycosidation is a kinetically controlled process, optimal reaction times must be always found to prevent excessive product hydrolysis. As shown in Table 2, maximum yields of *n*-hexyl and *n*-heptyl β-D-galactopyranoside were obtained after 1 h and 2 h, respectively.

Again, the isolation of the corresponding β-D-galactopyranosides was very simple due to their extractability into the organic phase constituted by the corresponding alcohol. After the phase separation, the unreacted D-lactose and the active enzyme remain in the buffer. Therefore, replacing the organic phase with another portion of alcohol may result in the continuation of the reaction and this second step can double the chemical yield of the desired D-galactoside. To verify this idea, we carried out two-step transgalactosidation with different reaction time sequences (Table 3). As expected, the overall product yields were significantly higher as compared to the one-step procedures, reaching 40% in the case of *n*-hexyl β-D-galactopyranoside. The two-step approach may thus generally serve as a successful strategy in enzymatic glycosidation.

CONCLUSION

The aim of this paper was to improve the enzymatic preparation of alkyl glycosides as concerns the product yields and the price of the catalyst. Kernel meal from apricot as a cheap source of β-glucosidase was found comparable to almond meal in respect of the yield of *n*-hexyl β-D-glucopyranoside prepared by reverse hydrolysis from D-glucose. The yields of *n*-hexyl β-D-galactopyranoside and *n*-heptyl β-D-galactopyranoside were

Table 2. Enzymatic preparation of *n*-hexyl β-D-galactopyranoside and n-heptyl β-D-galactopyranoside by transgalactosidation from D-lactose

	n-Hexanol			*n*-Heptanol			
Time (h)	1	3	6.5	1	2	3.5	6.5
Chemical yield (%)	11.4	10.2	5.9	5.4	8.9	8	5.8

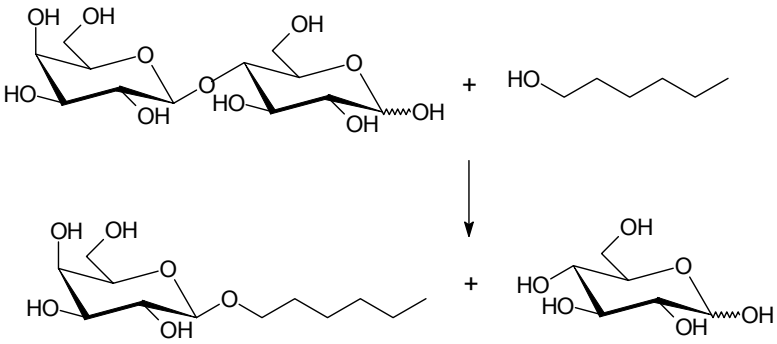

Figure 2. Transgalactosidation catalysed by commercial β-galactosidases

Table 3. Two-step enzymatic preparations of n-hexyl β-D-galactopyranoside and n-heptyl β-D-galactopyranoside by transgalactosidations from D-lactose

	Alcohol/Enzyme				
	n-hexanol fungal lactase			*n*-heptanol lactase F	
Time (h)	0.5 + 0.5	0.75 + 0.75	1 + 1	2 + 2	4 + 24
Chemical yield (%)	24	40	17	14	11.7

significantly improved by two-step transglycosidation from D-lactose using commercial galactosidases after optimisation of the reaction times.

References

Amalric C., Lecocu-Michel N. (1997): Concentrate comprising alkyl glycoside mixture and fatty alcohol and corresponding methods of use. US Patent 5670471.

Bradford M.M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, **72**: 248–254.

Garcia M.T., Ribosa L., Campos E., Sanchez Leal J. (1997): Ecological properties of alkyglucosides. Chemosphere, **35**: 545–556.

Lu W.-Y., Guo-Qiang Lin G.-Q., Yu H.-L., Tong A.-M., Xu J.-H. (2007): Facile synthesis of alkyl β-D-glucopyranosides from D-glucose and the corresponding alcohols using fruit seed meals. Journal of Molecular Catalysis B: Enzymatic, **44**: 72–77.

Meezan A., Pillion D.J. (1997): Absorption enhancers for drug administration. US Patent 5661130.

Okahata Y., Mori T. (1998): Transglycosylation catalyzed by a lipid-coated β-D-galactosidase in a two-phase aqueous-organic system. Journal of Molecular Catalysis B: Enzymatic, **5**: 119–123.

Tong A.M., Lu W.Y., Xu J.H., Lin G.Q. (2004): Use of apple seed meal as a new source of β-glucosidase for enzymatic glucosylation of 4-substituted benzyl alcohols and tyrosol in monophasic aqueous-dioxane medium. Bioorganic and Medicinal Chemistry Letters, **14**: 2095–2097.

van Rantwijk F., Woudenberg-van Oosterom M., Sheldon R.A. (1999): Glycosidase-catalysed synthesis of alkyl glycosides. Journal of Molecular Catalysis B: Enzymatic, **6**: 511–532.

Yu H.-L., Xu J.-H., Lu W.-Y., Lin G.-Q. (2007): Identification, purification and characterization of β-glucosidase from apple seed as a novel catalyst for synthesis of *O*-glucosides. Enzyme and Microbial Technology, **40**: 354–361.

Yu H.-L., Xu J.-H., Lu W.-Y., Lin G.-Q. (2008): Environmentally benign synthesis of natural glycosides using apple seed meal as green and robust biocatalyst. Journal of Biotechnology, **133**: 469–477.

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