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Novel reaction systems for the synthesis of *O*-glucosides by enzymatic reverse hydrolysis

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Abstract—Our studies are presented to replace alcohols as solvents in reverse hydrolytic reactions catalyzed by immobilized β-glucosidase to synthesize *O*-substituted β-D-glucopyranosides in preparative-scale. We found that 1,2-diacetoxyethane is a suitable solvent and *O*-alkyl or aryl β-D-glucosides were synthesized in moderate yields (after isolation 12–19%). In these reactions proportion of glucose and glucosyl acceptor hydroxy compounds was 1:20. We suggest that 1,2-diacetoxyethane can be useful not only for alcohols but for other glucosyl donor compounds unsuitable for the role of solvent (e.g., phenols) in the synthesis of *O*-β-D-glucosides by reverse hydrolysis.
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1. Introduction

It is known that enzymes are important catalysts in non-conventional media.[1] In chemical, pharmaceutical and food industry different hydrolases—proteases, esterases and glycosidases are used to catalyze the synthesis of biologically active peptides, esters and glycosides. In aqueous solutions, enzymatic hydrolytic reactions catalyzed by hydrolases are practically irreversible, but hydrolysis can be converted to synthesis by reducing the water content of the medium via using organic solvents—this is reverse hydrolysis.[2] Stereoselectivity and regioselectivity in enzymatic reactions are generally higher than in chemical syntheses.[3] In the presence of organic solvents, the solubility of substrate and/or product may be improved but the solubility, stability and activity of some enzymes can be diminished,[4] therefore immobilized enzymes are often used.[5] In most cases, some water is required to maintain the native conformation of the enzyme.[6]

Environmentally friendly *O*-alkyl glucosides are non-ionic and non-toxic surfactants[7] with antimicrobial ability and biodegradability.[8,9] Some of them are used in the detergent, food and pharmaceutical industry.[10,11] The synthetic activity of β-glucosidase in reverse hydrolytic processes was tested by Vulfson[12] then later by Laroute and Willemot.[13]

Keywords: β-Glucosidase; Immobilization; Enzyme reaction in non-conventional media; Reverse hydrolysis; *O*-Glucosylation; 1,2-Diacetoxyethane.

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Earlier we found that native glucosidases have significant *O*-glucosylation activity using glucose and different alcohols, but upscaling of the reactions reduced yields sharply because of heterogenity of reaction mixtures and enzyme deactivation in organic media. Yields were enhanced when immobilized forms of glucosidases on a modified polyacrylamide-type bead support (Acrylex C-100) were used.[14] It was a general supposition in the literature that alcohols have to be both glucosyl acceptors and solvents in these glucosylation reactions.[13]

Recent results of our study are presented to replace alcohols in solvent function in preparative-scale reverse hydrolytic synthesis of *O*-substituted β-D-glucopyranosides.

2. Results and discussion

The study of synthetic reactions was connected with the examination of enzyme deactivation in these reactions. The water content of the mixtures was chosen on the basis of our earlier results.[14] Deactivation of immobilized β-glucosidase was characterized in two different ways. The effect of reverse hydrolytic reaction mixtures on enzyme activity was characterized by immediate deactivation when the enzyme activities in different reaction mixtures were compared to the original enzyme activity measured in aqueous mixture (it was taken as hundred percent). The effect of reverse hydrolytic reaction mixtures on enzyme stability was characterized by deactivation kinetics when the loss in enzyme activity during reverse hydrolysis was compared to the enzyme activity at the start of the reaction (immediate deactivation value was taken as hundred percent).

2.1. Stability of immobilized β-glucosidase in alcohol–organic solvent mixtures of different water content

Different organic solvents as co-solvents were tested: hydrophilic aprotic solvents (e.g., dimethylformamide), ethers (e.g., dioxane), ketones (e.g., acetone), hydrocarbons (e.g., hexane), esters (e.g., ethyl acetate, 1,2-diacetoxy-ethane) and other solvents (e.g., acetonitrile). Immediate deactivation and deactivation kinetics of immobilized β-glucosidase were tested in reaction mixtures containing different alcohols of water content 5–10–15% and organic solvent content 0–5–50%. Generally the organic solvents tested decreased stability of immobilized β-glucosidase, moreover most of them caused an immediate and complete loss in enzyme activity. However, we found that in the presence of 1,2-diacetoxyethane in reaction mixtures the enzyme activity values of immobilized β-glucosidase (Table 1) were reduced only to one-half and its stability values (Table 2) were similar to data in pure alcohols.

Table 1. The immediate deactivation of immobilized β-glucosidase (expressed in residual activity %) in alcohol–1,2-diacetoxyethane mixtures

Alcohol (5–15% water content in the reaction mixture)	1,2-Diacetoxyethane content %		
	0	5	50
n-butanol	52±5	33±5	25±6
n-pentanol	42±4	27±5	22±5
n-hexanol	65±5	37±5	30±6
Cyclohexanol	71±7	38±5	28±7

The activity of the enzyme in aqueous reaction mixture was taken as hundred percent. For the mixtures of different water content mean values are given and differences (±) are presented.

Table 2. Residual activity (%) of immobilized β-glucosidase in alcohol–1,2-diacetoxyethane mixtures at the end of six-day reverse hydrolytic reaction at room temperature

Alcohol (5–15% water content in the reaction mixture)	1,2-Diacetoxyethane content %		
	0	5	50
n-butanol	42±4	40±4	32±6
n-pentanol	42±5	38±4	33±6
n-hexanol	43±6	41±5	34±8
Cyclohexanol	41±4	39±5	35±7

The activity of the enzyme at the start of the reaction was taken as hundred percent.For the mixtures of different water content mean values are given and differences (±) are presented.

Organic solvents may affect the properties of enzymes in several ways including interactions with the hydration layer essential for catalysis and proper folding as well as alteration of protein conformation by direct interaction with protein solvation sites either hydrophobic or hydrogen bonding.[4] It is known that glucosidases show higher affinity for hydrophobic substrates than hydrophilic ones. In transglucosylation reactions, hydrophobic acceptors like alcohols and diols produce glucosides but very hydrophilic alcohols, for example, glycerol cannot not be transglucosylated.[15] It was presumed that this kind of co-solvent can weaken the hydrophobic interaction between substrate alcohol and amino acid side chains of the enzyme binding sites.[16] It is attributed to the hydrophobic character of 1,2-diacetoxyethane (on the basis of log P values slightly stronger than that of the alcohols examined) that the decrease in hydrophobic enzyme–substrate interactions was small.

Rate constants (k) ($10^{-3}\,h^{-1}$) of deactivation kinetics of immobilized β-glucosidase during reverse hydrolysis were determined for hexanol-1,2-diacetoxyethane–water mixtures at room temperature. They were obtained from the slope of the regression in the plots of lg residual enzyme activity versus reaction time [$\lg(A/A_o)=-kt$] (detailed only for hexanol of 50% 1,2-diacetoxyethane content in Fig. 1). The results compiled in Table 3 and Figure 1 suggest first order kinetics for inactivation.

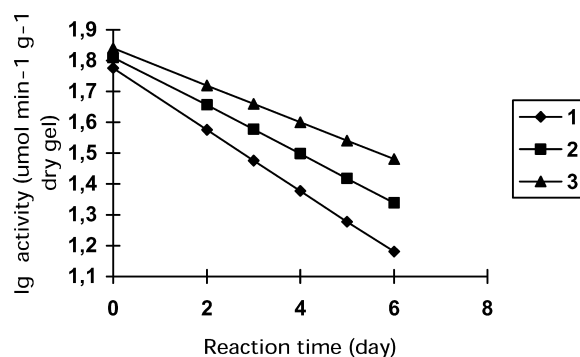

Figure 1. Plot of the logarithm of the residual enzyme activity as a function of reaction time in deactivation of immobilized β-glucosidase during reverse hydrolysis in hexanol with 50% 1,2-diacetoxyethane content at room temperature. Water content 5% (1), 10% (2), 15% (3).

Table 3. Rate constants (k) ($10^{-3}\,h^{-1}$) of deactivation of immobilized β-glucosidase during reverse hydrolysis in hexanol-1,2-diacetoxyethane–water mixtures at room temperature

Content of mixture	Water content		
	5	10	15
(1)	100	116.4	144.5
(2)	98	107	126.5
(3)	72	92.6	120.5

Content of mixture (1) hexanol, (2) hexanol with 5% 1,2-diacetoxyethane content, (3) hexanol with 50% 1,2-diacetoxyethane content.

2.2. Reverse hydrolysis catalyzed by immobilized β-glucosidase in alcohol–organic solvent mixtures of different water content

There are only a few published data in the literature for replacing glucosyl acceptor alcohols as solvents in reverse hydrolysis, for example, *O*-octyl-β-D-glucopyranoside was obtained in 8% yield using a 60:30:10 (v/v) *tert*-butanol–octanol–water mixture.[17] The yields of *O*-glucosylations with glucose as glucosyl donor and different alcohols both as glucosyl acceptors and solvents in reverse hydrolytic processes with different water content catalyzed by immobilized β-glucosidases in absence and presence of different organic solvents as co-solvents were studied. In most of the cases of the organic solvents examined as co-solvents, the synthetic reactions (e.g., dimethylformamide, dioxane, acetone, hexane, ethyl acetate and acetonitrile) practically failed. The yields in alcohol-1,2-diacetoxyethane

(1:1) mixtures were between 30–58% on the basis of HPTLC method. The best results were found for all alcohols studied at 10% water content after 6 days at room temperature (Table 4). We ascribe the efficiency of 1,2-diacetoxyethane as co-solvent in *O*-glucosylation reactions by reverse hydrolysis to its hydrophobic character (slightly stronger than that of the alcohols examined) and water solubility (143 mg ml^{-1}). We found no direct correlation between stability data of glucosidases or yield of reverse hydrolyses and different physical parameters (e.g., dielectric constant, log *P*, solubility in water) of both alcohols and organic solvents or 1,2-diacetoxyethane content of the reaction mixtures.

Table 4. Maximum yields (%) of *O*-glucosylation reactions catalyzed by immobilized β-glucosidase in different alcohol–1,2-diacetoxyethane mixtures (% water content) at room temperature (reaction time 6 days) on the basis of HPTLC method

Alcohol	1,2-Diacetoxyethane content (%)		
	0	5	50
n-butanol	45 (15)	43 (15)	43 (10)
n-pentanol	48 (15)	47 (15)	46 (10)
n-hexanol	57 (15)	58 (15)	60 (10)
Cyclohexanol	52 (15)	54 (10)	56 (10)

2.3. In preparative-scale reactions in 1,2-diacetoxyethane of 10% water content as solvent

We found that in reverse hydrolysis catalyzed by immobilized β-glucosidase to synthesize *O*-alkyl β-D-glucosides, alcohols as solvents can be replaced by 1,2-diacetoxyethane therefore the synthesis of *O*-alkyl-β-D-glucosides in presence of 1,2-diacetoxyethane in preparative-scale was attempted. The reaction mixtures were evaporated in vacuo on a larger scale (boiling point of 1,2-diacetoxyethane is only 79–81 °C at 1.45×10^{-3} Pa) and glucosides were isolated by thick layer chromatography of residues. We prepared not only *O*-alkyl β-D-glucosides which were synthesized earlier in alcohols as solvents[14] but some other *O*-alkyl and aryl β-D-glucosides were formed by reverse hydrolysis catalyzed by immobilized β-glucosidase in 1,2-diacetoxyethane in preparative-scale. *O*-Aryl β-D-glucosides have never been synthesized by reverse hydrolysis.

3. Conclusion

We found that 1,2-diacetoxyethane is a suitable solvent to replace alcohols as solvents in reverse hydrolytic reactions catalyzed by immobilized β-glucosidase to synthesize *O*-subsituted (alkyl or aryl) β-D-glucosides. In these reactions, proportion of glucose and glucosyl acceptor hydroxy compounds was 1:20. We suggest that 1,2-diacetoxyethane can be useful not only for alcohols but for other glucosyl donor compounds unsuitable for the role of solvent (e.g., phenols) in the synthesis of *O*-β-D-glucosides by reverse hydrolysis.

4. Experimental

4.1. Materials

β-Glucosidase (G 0395) from almonds (EC 3.2.1.21), *n*-butanol, *n*-pentanol, *n*-hexanol and cyclohexanol were SIGMA products. Other hydroxy compounds, D-glucose, D-cellobiose, 1,2-diacetoxyethane (ethylene glycol diacetate) and other chemical materials were purchased from FLUKA. β-Glucosidase was immobilized on a modified polyacrylamide-type bead support (Acrylex C-100) possessing carboxylic groups by the carbodiimide method described earlier.[14] Immobilized β-glucosidase showed an activity per gram dry gel of 153 μmol min^{-1} (V_{max} 233 μmol min^{-1}, K_M 0.1 M). Binding capacity of the support was 6.2±0.3 mequiv. per gram dry gel, particle size 100–320 μm. Activity of β-glucosidase was measured according to the literature[18] with substrate cellobiose (0.25 mol l^{-1}) in sodium acetate buffer (0.1 mol l^{-1}, pH 5.1).

4.2. General procedures for reverse hydrolysis

4.2.1. In different alcohol–organic solvent mixtures of different water content. Reaction mixtures (0.5 ml) containing immobilized β-glucosidase (3 mg) and glucose (9 mg, 0.05 mmol) in different alcohol–organic solvent mixtures of 5–10–15% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed twice with distillated water (1 ml). Combined filtrate was analysed for glucose and *O*-alkyl glucoside content and the activity of washed immobilized enzyme was determined.

Concentration of unreacted glucose was measured by enzymic analysis using hexokinase, glucose-6-phosphate dehydrogenase and NADP (Sigma) then mixtures were evaporated to dryness in vacuo. The *O*-alkyl glucoside content of residues was analysed by Chrompress LABOR-MIM (Hungary) HPTLC[19] and Ultrascan XL LKB laser densitometer. Yields were determined on the basis of HPTLC method.

4.2.2. In different alcohol–1,2-diacetoxyethane mixtures of different water content. Reaction mixtures (0.5 ml) containing immobilized β-glucosidase (3 mg) and glucose (9 mg, 0.05 mmol) in different alcohol–1,2-diacetoxyethane mixtures of 5–10–15% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed twice with distillated water (1 ml). Combined filtrate was analysed for glucose and *O*-alkyl glucoside content and the activity of washed immobilized enzyme was determined.

Concentration of unreacted glucose was measured by enzymic analysis using hexokinase, glucose-6-phosphate dehydrogenase and NADP (Sigma) then mixtures were evaporated to dryness in vacuo. The *O*-alkyl glucoside content of residues was analysed by Chrompress LABOR-MIM (Hungary) HPTLC[19] and Ultrascan XL LKB laser

densitometer. Yields are given on the basis of HPTLC method (Table 4).

4.2.3. In preparative-scale reactions in 1,2-diacetoxyethane of 10% water content as solvent. Reaction mixtures (5 ml) containing immobilized β-glucosidase (30 mg), glucose (0.18 g, 1 mmol) and hydroxy compound (20 mmol) in 1,2-diacetoxyethane of 10% water content were shaken on IKA-VIBRAX-VXR shaker at speed 200 min^{-1} at room temperature for 6 days. Immobilized enzyme was filtered off and washed three times with distilled water (5 ml). The isolation of glycosides by evaporation of filtrate in vacuo and preparative layer chromatography of residue on Kieselgel PF_{254} layer by chloroform–ethyl acetate–methanol mixtures as eluants gave pure *O*-alkyl and aryl β-D-glucosides.

4.3. Structure identification

All of *O*-alkyl and aryl β-D-glucosides synthesized in preparative-scale are known in the literature and they were identified by the authentic samples. Their optical purity was controlled by their ^{1}H-NMR spectra according to the literature[20] (δ 4.50–4.56 ppm 1 H, d, 1-H). ^{1}H-NMR spectra were taken on a Varian EM-390 90 MHz instrument using TMS as internal standard in DMSO-d_6. Yields after isolation are given in parentheses.

O-*n*-Butyl β-D-glucopyranoside[15] (15%);
O-*n*-Pentyl β-D-glucopyranoside[21] (17%);
O-*n*-Hexyl β-D-glucopyranoside[11] (19%);
O-Cyclohexyl β-D-glucopyranoside[21] (19%);
O-*n*-Octyl β-D-glucopyranoside[10] (18%);
O-*n*-Nonyl β-D-glucopyranoside[10] (16%);
O-*n*-Decyl β-D-glucopyranoside[10] (12%);
O-Phenyl β-D-glucopyranoside[22] (13%);
O-4-Nitrophenyl β-D-glucopyranoside[23] (11%).

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