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Enzymatic preparation of melibiose and alkyl β-D-fructofuranosides by commercial lactase

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ABSTRACT

Lactozyme 3000L was found to possess β-fructofuranosidase activity and catalyzed hydrolysis of raffinose to melibiose and formation of alkyl β-D-fructofuranosides from sucrose. The total chemical yield of melibiose was 95.5%, yields of alkyl β-D-fructofuranosides ranged from 5.2 to 20.2% depending on the structure of the acceptor. Alcohols larger than butanol were not glycosylated.

1. Introduction

Glycanases and glycosidases are enzymes catalyzing hydrolysis of glycosidic bonds and are widely used in industrial processing of saccharides. Invertases (β-fructofuranosidases) are primarily used in food industry for production of glucose–fructose syrups although this process can hardly compete with production of high fructose syrups by glucose isomerisation. These enzymes are however under focus of scientists due to their potential in production of fructooligosaccharides as a dietary supplement and there is a constant demand for new cheap sources of this class of enzymes [1–5].

Lactases (β-galactosidases) find industrial use in removal of lactose from milk [6], processing of whey to ethanol [7] or pure galactose [8] or in production of galactooligosaccharides [9–12]. Less commonly, they find use in preparation of alkyl β-D-galactopyranosides [13–15], structured biologically relevant oligosaccharides [16,17] and precursors of glycoconjugates [18].

To keep prices of industrial enzymes competitive, their purification is minimized and the biocatalysts are usually sold as rather crude enzyme cocktails with one main activity and varying levels of other enzymes like proteases, esterases and glycosidases. Our group focusses on identification of useful side-activities in commercial enzymes and use them in syntheses of various saccharidic derivatives [14,19–21].

Recently, we have found β-fructofuranosidase activity in two commercial β-galactosidases - Lactase F (Amano, from *Aspergillus oryzae*), and Lactozyme 3000L (Novozymes, from *Klyuveromyces lactis*) and decided to test Lactozyme as a source of invertase in hydrolysis of raffinose to melibiose and in alcoholysis of sucrose to alkyl β-D-fructofuranosides.

Raffinose is a non-reducing trisaccharide consisting of D-galactopyranose, D-glucopyranose and D-fructofuranose. The whole molecule can be supposed as a conjuction of mellibiose and sucrose with glucose in their intersection. Hydrolysis of raffinose by α-galactosidase forms galactose and sucrose while invertase produces mixture of fructose and melibiose.

Long-chain alkyl or alkenyl C_6–C_{24} fructofuranosides may serve as nonionic surfactants for cosmetics and dish-cleaners [22] while water soluble alkyl β-D-fructofuranosides may be used as substrates for invertase assay [23]. Such molecules may find also use as chiral building blocks due to the presence of four chiral centers and two primary hydroxyls which are considerably more reactive over secondary hydroxyls in position 3-and 4-.

There are generally two approaches to one-step enzymatic synthesis of glycosides: reverse hydrolysis and transglycosidation. Reverse hydrolysis is a thermodynamically controlled process in which a free saccharide slowly reacts with an alcohol until an equilibrium is reached between starting substances and the target glycoside. Besides low reaction rate, the process usually suffers from low yields and complicated purification of the product,

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since higher concentrations of substrates are required. To prevent formation of undesired anomers, the enzymes must be free of glycosidases with the opposite anomer specificity. Reverse hydrolysis is advantageous especially in reactions where cheap starting saccharide is used for glycoside formation.

Transglycosidation is a kinetically controlled process employing partial transferase activity of glycosidases. The enzyme catalyzes transfer of glycosyl unit from the non-reducing end of oligosaccharide, polysaccharide or other glycoside to the alcohol or other acceptor (saccharide, amine, thiol, etc.). After reaching a maximum concentration, the product hydrolysis prevails over its synthesis. Reaction time is therefore a crucial factor in an effective glycoside preparation by transglycosidation. Comparing to reverse hydrolysis, the transglycosidation is faster, requires lower substrate concentrations and gives usually higher yields. Anomeric purity of product is controlled by substrate choice, so contamination of biocatalyst by glycosidase with opposite anomer specificity does not intrigue the reaction. The level of transferase activity is strongly varying among glycosidases and free monosaccharides are formed as side-products arising from hydrolysis. Since only part of dissolved free fructose is in the β-fructofuranoside form (published data range from 19.5% [24] to cca 27% [25]), the invertase catalyzed synthesis of fructofuranosides proceeds as the transfructosidation with sucrose as the β-fructofuranosyl donor.

2. Experimental

2.1. Analytical methods

HPLC measurements were performed on an assembly from Watrex Slovakia consisting of isocratic pump, RI detector, column owen and Rheodyne injector with 20 μL injection loop. Free sugars were assayed on Polymer IEX H^+ column (250 × 8 mm) from Watrex, eluted with 9 mM H_2SO_4 (0.5 mL/min.) at 40 °C. Glycosides were estimated on MZ Aqua Perfect column (250 × 4 mm, particle size 5 μm) from MZ Analysentechnik eluted with deionized water at 40 °C.

Transglycosidations were monitored by TLC on silica gel plates with chloroform/methanol (3/1, v/v) as eluent. The compounds were detected by charring the plates with 10% (v/v) ethanolic solution of H_2SO_4 and heating at ca. 200 °C.

Paper chromatography was performed by the descending method on Whatman No. 1 paper using 8:2:1 ethyl acetate–pyridine–water as the mobile phase. The spots of compounds were detected with alkaline silver nitrate.

Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 °C. ^{1}HNMR spectra were recorded on 400 MHz with Varian MR (Me_4Si as internal standard). ^{13}C NMR spectra were recorded at 100 MHz and chemical shifts are referenced to CD_3OD as internal standard.

2.2. Materials

Sugars, alcohols and buffer components were purchased from Lachema, Brno, Czech Republic, Acros Organics, Geel, Belgium and Merck, Darmstadt, Germany. Thin-layer chromatography was performed on precoated silica gel 60 F254 plates (0.25 mm, Merck). Silica gel (0.035–0.070 mm, pore diameter ca. 6 nm, Acros Organics) was employed for column chromatography.

2.3. β-Fructofuranosidase assay

The reaction mixture containing 15 mL 4% (w/v) sucrose in 0.1 M acetate buffer (pH 5.0) and 50 μL of diluted enzyme was incubated at 40 °C. 50 μL aliquots were periodically withdrawn in 10 min intervals and mixed with 950 μL of 9 mM H_2SO_4 followed by vortexing and filtration through 0.22 μm syringe filter. The concentration of released fructose was estimated by HPLC. One invertase unit corresponds to an amount of enzyme releasing 1 μm of fructose per one minute. The actual activity of invertase in Lactozyme was found to be 530.25 U/mL.

2.4. Kinetics of raffinose hydrolysis

Optimisation of raffinose hydrolysis was performed in 15 mL of 0.1 M acetate buffer (pH 5.0) containing 1, 2, or 3% (w/v) raffinose pentahydrate, respectively. The hydrolysis was started by addition of 50 μL of diluted Lactozyme (10.6 U of invertase) and kept stirred at 40 °C. 50 μL aliquots were withdrawn in predefined time intervals and processed the same way as in invertase assay. Levels of raffinose, melibiose and fructose were estimated by HPLC.

2.5. Preparation of melibiose

D-Raffinose pentahydrate (4.5 g) was dissolved in 150 mL 0.1 M acetate buffer (pH 5.0) followed by addition of 200 μL Lactozyme (106 U of invertase). The whole mixture was stirred at 40 °C for 24 h. The reaction was stopped by boiling for 10 min. The mixture was concentrated on rotavap, desalted on ion-exchangers and evaporated to dryness. The dry rest was redissolved in methanol (20 mL) and left to crystallize to get 2 g of melibiose (77.2%). The mother liquor containing mixture of melibiose and fructose was further fractioned on Amberlite IRA 120 in Ca^{2+} cycle (100 × 3 cm) equilibrated and eluted with 48% (v/v) ethanol to give 0.5 g (18.3%) of melibiose hydrate and 1.2 g of fructose. The structure of melibiose was confirmed by paper chromatography, HPLC, NMR and optical rotation $[\alpha]_D^{20}$ +135.0 (c = 1, MeOH).

2.6. Alcoholysis of sucrose by aliphatic alcohols

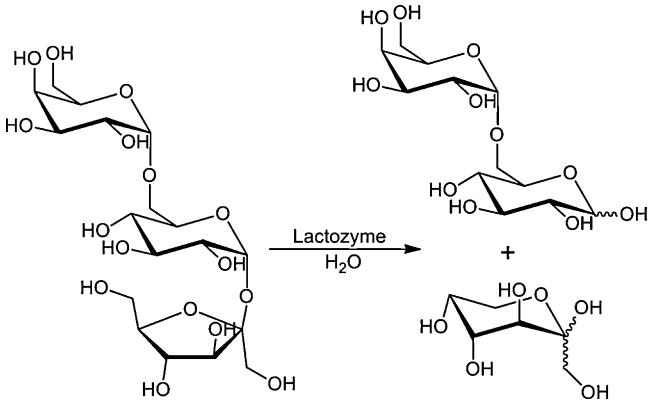
40 μL of diluted Lactozyme (8.5 U of invertase) was added to mixture of 2 mL of 3% (w/v) sucrose in 0.1 M acetate buffer (pH 5.0) and 0.5 mL of an alcohol (ethanol, n-propanol, n-butanol, n-hexanol or n-octanol) and whole mixture was shaken on VIBRAMAX 100 shaker (Heidolf) at 750 rpm and 40 °C. The course of the reaction was monitored by thin layer chromatography.

2.7. Optimisation of sucrose ethanolysis

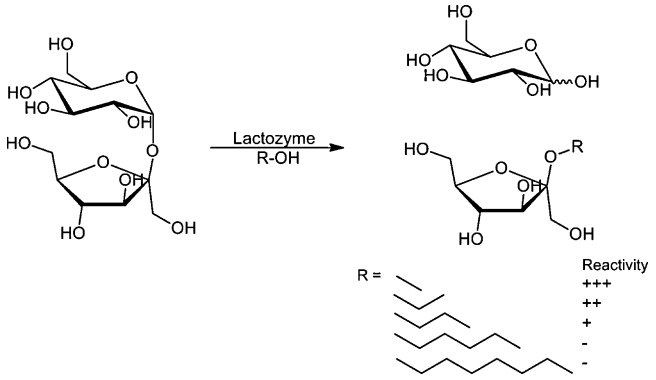
Vials containing 2 mL of reaction mixtures with predefined concentration of sucrose (2, 3, 4, 5, 6, 7, 8, 9, and 10%) and ethanol and 8.5 U of Lactozyme invertase in sodium acetate buffer pH 5 were shaken on VIBRAMAX 100 shaker (Heidolf) at 750 rpm and 40 °C (sucrose optimisation) or 37 °C (ethanol optimisation, full kinetics). Either after 1 h, or in predefined time intervals, 100 μL of the reaction mixture was mixed with 900 μL of 0.2 M Na_2CO_3, vortexed, passed through nylon syringe microfilter and analyzed by HPLC.

2.8. Preparative alcoholysis

Solution of 80 mL 0.1 M sodium acetate buffer pH 5 comprising 8 g of sucrose was mixed with 20 mL of alcohol (ethanol, n-propanol or n-butanol) and pretempered to 37 °C. Diluted Lactozyme (2 mL, 424 U of invertase) was added and the reaction mixture was stirred for 2 (ethanol), 4 (n-propanol) or 24 h (n-butanol). The reaction mixtures were concentrated on rotavap and desalted by small amounts of ion exchange resins. Concentrated solutions were applied on Dowex 1 × 4 column (150 × 2.6 cm) in OH^- form equilibrated and eluted by water to remove excess of free saccharides. Fractions comprising products were collected, evaporated to dryness with silica gel and applied on silica gel column equilibrated



Scheme 1. Hydrolysis of raffinose by Lactozyme 3000L.



Scheme 2. Alcoholysis of sucrose by Lactozyme 3000L.

and eluted with chloroform/methanol (3/1, v/v). Fractions comprising pure products were collected and evaporated to dryness to give ethyl β-D-fructofuranoside (0.968 g, 20.2%), $[\alpha]_D^{20}$ −30.0 (c = 1, MeOH), n-propyl β-D-fructofuranoside (0.526 g, 9,6%), $[\alpha]_D^{20}$ −36.0 (c = 1, MeOH), and n-butyl β-D-fructofuranoside (0.290 g, 5.2%), $[\alpha]_D^{20}$ −31.0 (c = 1, MeOH). The NMR spectra of products were in accordance with values reported previously [26].

3. Results and discussion

3.1. Hydrolysis of raffinose

Lactozyme 3000L can be very conveniently used for preparation of melibiose (Scheme 1). Raffinose up to concentration 30 g/L was reliably hydrolyzed within 24 h (Fig. 1). Higher concentrations of the starting saccharide were not used to prevent formation of oligosaccharides arising from transfructosidation since hydrolysis of sucrose at concentrations above 4% - tested within development of assay method for invertase - produced higher saccharides observable at HPLC chromatograms (data not shown). The total yield of melibiose was 95.5%.

3.2. Reactivity of invertase in sucrose and raffinose alcoholysis

In the initial studies, specificity of Lactozyme in sucrose alcoholysis (Scheme 2) was monitored by tlc as described in Section 2.7. The enzyme catalyzed the fructosyl transfer only when water-miscible alcohols (ethanol, n-propanol), or n-butanol (solubility in water approx. 1 mol/L) were used as acceptors. Hexanol and octanol, forming typical two-phase system (solubility in water 0.06 or 2×10^{-6} mol/L, respectively) were not glycosylated. Reaction with ethanol very quickly produced ethyl β-D-fructofuranoside, hydrolysis of which became evident after 1 h. The product disappeared within 24 h. Reaction with butanol was slow and the product was present in the reaction mixture even after 7 days. Alcoholysis of raffinose gave only traces of fructosides. The concurrent preparation of fructofuranosides and melibiose with Lactozyme is therefore not feasible.

To optimize the sucrose ethanolysis, varying concentrations of sucrose and ethanol were reacted and productivity of ethyl β-D-fructofuranoside was evaluated after 1 h by HPLC (Figs. 2 and 3). The optimum sucrose concentration to achieve maximum product content and maximum product yield was 8% (Fig. 2), with conversions ranging from 25 to 35%. The optimum concentration of ethanol was found to be 20% (v/v). To reduce the risk of alcohol evaporation in long-term experiments, the optimisation of ethanol content was performed at 37 °C instead of 40 °C. Comparing to the reaction conditions within the preliminary tlc screenings (sucrose concentration 3% (w/v)), the ethanolysis under optimum sucrose concentration reached the maximum product level later - between 2 and 4 h (Fig. 4). This finding was important for effective preparation of ethyl β-D-fructofuranoside.

3.3. Preparative fructosylations of alcohols

The transfructosidations of ethanol, n-propanol and n-butanol were repeated in preparative scale. Reactions with propanol and

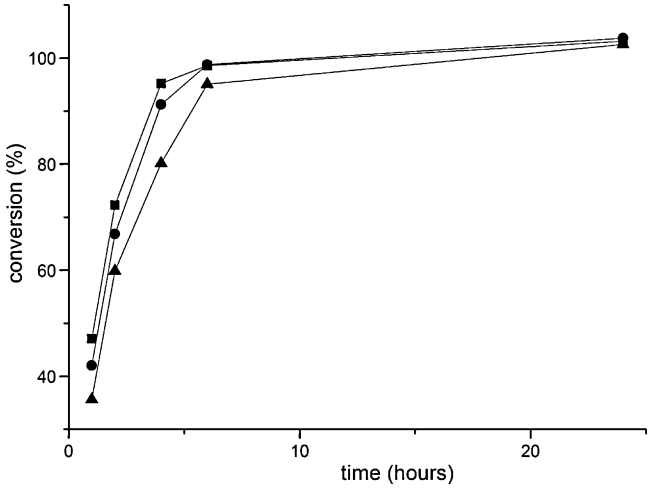


Fig. 1. Hydrolysis of raffinose to melibiose by Lactozyme 3000L. Starting concentrations of raffinose 1% (w/v) (-■-); 2% (w/v) (-●-) and 3% (w/v) (-▲-).

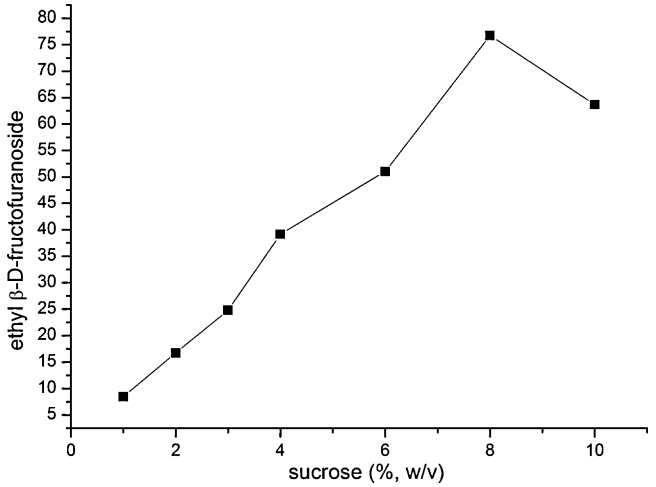


Fig. 2. Productivity of ethyl β-D-fructofuranoside after 1 h by Lactozyme 3000 L in various starting concentrations of sucrose. Ethanol concentration 20% (v/v).

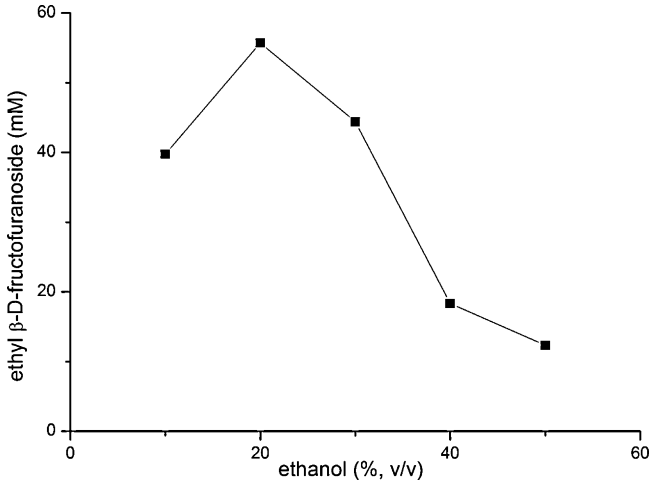


Fig. 3. Productivity of ethyl β-D-fructofuranoside after 1 h by Lactozyme 3000 L in various starting concentrations of ethanol. Sucrose concentration 3% (w/v).

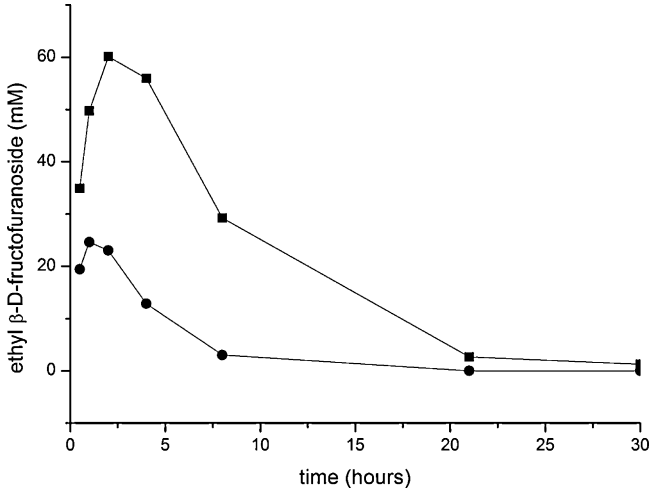


Fig. 4. Time course of sucrose ethanolysis by Lactozyme 3000 L at sucrose concentration 3% (w/v) (-●-) and 8% (w/v) (-■-). Ethanol concentration 20% (v/v).

butanol were not optimized according to alcohol content and conditions from the synthesis of ethyl β-D-fructofuranoside were adopted in these experiments under prolonged reaction times. The chemical yields of products 1–3 follow the trend of decreasing fructosylation productivity with extending the chain length of the respective alcohol.

4. Conclusion

Lactozyme 3000 L may be conveniently used for preparation of melibiose from raffinose in 95.5% yield. Moreover, the enzyme catalyzed synthesis of short alkyl β-D-fructofuranosides by alcoholysis of sucrose in yields ranging from 5.2% to 20.2% depending on the length of alcohol. Lactozyme is therefore a versatile catalyst which, in addition to its main lactase activity, may be used also for hydrolysis and formation of glycosidic bonds other than β-galactosides due to the presence of relatively high level of invertase. Control of the reaction is performed through the choice of substrate and reaction conditions.

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