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Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G)

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Abstract

A study on optimisation of the conditions for galactooligosaccharide (GOS) formation during lactose hydrolysis, produced by Lactozym 3000 L HP G, was carried out. The synthesis was performed during times up to 300 min at 40, 50 and 60 °C, pH 5.5, 6.5 and 7.5, lactose concentration 150, 250 and 350 mg/mL and enzyme concentration 3, 6 and 9 U/mL. The product mixtures were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). During the hydrolysis of lactose, besides glucose and galactose, galactobiose, allolactose and 6′ galactosyl lactose were also formed as a result of transgalactosylation catalysed by the enzyme. The effect of the reaction conditions was different in the formation of di- and the trisaccharide. Thus, the optimal conditions for galactobiose and allolactose synthesis were 50 °C, pH 6.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 300 min, whereas the best reaction conditions for 6′ galactosyl lactose production were 40 °C, pH 7.5, 250 mg/mL of lactose, 3 U/mL of enzyme and 120 min. These results show the possibility to obtain reaction mixtures with Lactozym 3000 L HP G, with different composition, depending on the assayed conditions.

Keywords: Galacto-oligosaccharides; Lactose; Lactozym 3000 L HP G

1. Introduction

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides, comprised of 2–20 molecules of galactose and one glucose (Miller & Whistler, 2000), which are recognised as prebiotics because they can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine (Sako, Matsumoto, & Tanaka, 1999). Other health benefits, such as reduction of the level of cholesterol in serum, colon cancer prevention and enhancement of calcium absorption have been also described (Perugino, Trincone, Rossi, & Moracci, 2004; Sako et al., 1999; Tuohy, Rouzaud, Brück, & Gibson, 2005). The confirmed health claims of GOS have significantly increased the public demand for foods containing GOS, particularly in Japan and Europe (Gaur, Pant, Jain, & Khare, 2006). For this reason, a lot of attention has been paid to GOS production, especially via enzymatic transgalactosylation since chemical synthesis of GOS is very tedious (Sears & Wong, 2001). GOS can be synthesised from lactose when this sugar acts as the acceptor and transgalactosylation is catalysed by the enzyme β-galactosidase (EC 3.2.1.23). However, if this acceptor is a water molecule, galactose is released through a hydrolysis reaction (Crittenden & Playne, 1996). The ratio of transferase and hydrolase activities of the enzyme affects the amount and nature of the formed oligosaccharides, since the enzyme source, the concentration and nature of the substrate and the reaction conditions

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(pH, temperature and time) are the main affecting factors (Gaur et al., 2006; Kim, Ji, & Oh, 2004; Mahoney, 1998). Although hydrolysis of synthesised oligosaccharides competes with transgalactosylation, the latter can be favoured at high lactose concentration, elevated temperature and lower water activity (Gaur et al., 2006). Hence, a deep knowledge of the time course of the reaction is required to determine the point of maximum yield of the desired products.

The yeast *Kluyveromyces lactis* is an important commercial source of β-galactosidases (β-D-galactohydrolase, EC 3.2.1.23) (Tello-Solis et al., 2005), with Lactozym 3000 L HP G being one of the most used enzymatic preparations. This enzyme, for its huge hydrolytic activity, has been used to produce lactose-free milk products. Moreover, during this hydrolysis the production of GOS via transgalactosylation has also attracted interest during the last few years. Thus, although a number of studies have been reported on the synthesis of GOS using β-galactosidase from *K. lactis*, most are focused on the formation of trisaccharides and small amounts of higher molecular weight oligosaccharides (Boon, Janssen, & Van't Riet, 2000; Bridiau, Taboubi, Marzouki, Legoy, & Maugard, 2006; Chockchaisawasdee, Athanasopoulos, Niranjan, & Rastall, 2005; Hung & Lee, 2002; Matsumoto et al., 1993) and there are few references on the formation of disaccharides other than lactose (Cheng et al., 2006; Maugard, Gaunt, Legoy, & Besson, 2003). As it is known, disaccharides such as lactulose possesses an important prebiotic character (Tuohy et al., 2002), therefore it is necessary to gain more insight on the formation not only of trisaccharides but also on the disaccharide fraction during transgalactosylation reaction. The present study deals with factors (temperature, pH, time and substrate and enzyme concentration) affecting the formation of the main disaccharides and trisaccharides during lactose hydrolysis, using β-galactosidase from *K. lactis* (Lactozym 3000 L HP G).

2. Materials and methods

2.1. Materials

Lactose monohydrate was supplied by Scharlau (Spain) and D-glucose by Sigma–Aldrich Co. (Germany). D-Galactose, melezitose monohydrate and *o*-nitrophenyl β-D-galactopyranoside (oNPG) were purchased from Fluka (Steinheim, Germany) and galactobiose (β-D-Galp-(1 → 6)-Gal) from Sigma (Steinheim, Germany). Soluble commercial preparation of β-galactosidase from *K. lactis* (Lactozym 3000 L HP G), was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

2.2. Enzyme characterisation

β-Galactosidase activity was measured using *o*NPG as the substrate. The hydrolysis of *o*NPG was assayed at 40 °C, using *o*NPG at 0.5 g L^{-1} in buffer solution (50 mM potassium phosphate buffer, containing 1 mM $MgCl_2$, pH 6.5). The hydrolysis products are galactose and *o*-nitrophenol (*o*NP) and the enzymatic mechanism is similar to that of lactose hydrolysis. Samples, of 1 mL, were withdrawn at different times. The reaction was stopped by adding 1 mL of 0.5 N H_2SO_4 to 0.44 mL of sample. Afterwards, 1.5 mL of 1 M $NaCO_3$ was added to develop the yellow colour due to the presence of *o*NP. This colour was measured spectrophotometrically at a wavelength of 420 nm. Lactozym 3000 L HP G expressed a β-galactosidase activity of 3205 U. One enzyme unit is defined as the amount of enzyme releasing 1 μmol of *o*NP per mL per minute at 40 °C, pH 6.5.

The amount of soluble protein was performed according to Smith et al. (1985) using the BCA™ Protein Assay Kit procured by Pierce (Illinois, USA). Bovine serum albumin (BSA) was used as standard. Specific activity (U/mg) of the enzyme was calculated from the relation of the activity units (U/mL) over protein concentration (mg/mL). The soluble protein content in the commercial enzyme extract was 36.3 mg/mL. Therefore, the enzyme expressed a specific activity of 88 U/mg.

2.3. Enzymatic synthesis of GOS

Lactose solutions were prepared in 50 mM potassium phosphate buffer containing 1 mM $MgCl_2$. Different reaction conditions were assayed: temperature (40, 50 and 60 °C), pH (5.5, 6.5, 7.5), lactose (150, 250 and 350 mg/mL) and enzyme (3, 6 and 9 U/mL) concentrations. Experiments were carried out in duplicate. Reactions were performed in eppendorfs incubated in an orbital shaker at 300 rpm. Samples were withdrawn at specific time intervals (30, 60, 120, 180, 240 and 300 min) and immediately immersed in boiling water for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent analysis.

2.4. Chromatographic determination of carbohydrates

The carbohydrate composition of the reaction mixtures was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), on a ICS2500 Dionex system consisting of GP50 gradient pump and ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Data acquisition and processing was performed with Chromeleon version 6.7 software (Dionex Corporation, Sunnyvale, CA). For eluent preparation, Milli-Q water, 50% (w/v) NaOH and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing with helium for 25 min.

Separations were performed following the Van Riel and Olieman method (1991) with some modifications. Elution was at room temperature on a CarboPac PA-10 column (4 mm × 250 mm) connected to a CarboPac PA-10 (4 mm × 50 mm) guard column. Eluent A (12.5 mM NaOH), eluent B (8 mM NaOAc), eluent C (125 mM