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Purification and properties of a β-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction[1]

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β-D-Galactosidase was extracted by ultrasonic treatment in the presence of Triton X-100. The transgalactosylase (TGase) was separated from other β-D-galactosidases and purified 1720-fold, as measured by the TGase assay. The enzyme was shown to be homogeneous on native PAGE. The complex nature of the enzyme was demonstrated by SDS/PAGE, with four major protein bands of 163, 170, 178 and 190 kDa. The molecular mass was estimated to be 362 kDa by gel chromatography, and the pI was 5.25. The purified enzyme was stable at temperatures below 45 °C and over the pH range from 6.5–8. Lactose hydrolysis by the purified enzyme and by *Bifidobacterium bifidum* cells took place at the same pH and temperature, at pH 6.5 and 37 °C respectively. For the TGase, however, *B. bifidum* cells exhibited specific galactosyltransferase activity at pH 4.5 and at 40 °C, even though the transgalactosylation (TG) reaction was preferentially established by the purified enzyme at pH 4.8 and 45 °C. The amount of galacto-oligosaccharides (GOS) could be as high as 29% of the total sugar, and maximum production was achieved when 60% of the initial lactose was transformed by *B. bifidum* cells. The K_m values of the purified β-D-galactosidase were 2 mM for p-nitrophenyl galactosidase and 13 mM for lactose. In the TG reaction, the Michaelis constant was 800 mM. Calcium and EDTA affected the TGase/hydrolase ratio. The purified enzyme exhibited a specificity towards glycosidic acceptors, and all tested β-D-galacto-oligosides can be used as galactosyl residue donor to produce GOS.

β-D-Galactosidase (EC 3.2.1.23) is an enzyme which is widespread in Nature [1] and is able to catalyse not only the hydrolysis of β-D-galactosidic linkages, but also the transgalactosylation (TG) reaction to produce oligosaccharides [2–14].

Transfer reaction products do not seem to have a predominant physiological role, even though allolactose formed by *Escherichia coli* galactosidase is a natural inducer of the *lac* operon [15]. Galacto-oligo-

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[3] Abbreviations used: pNP-Gal, *p*-nitrophenyl β-D-galactopyranoside; GOS, galacto-oligosaccharide; TG, transgalactosylation; TGase, transgalactosylase.

saccharides (GOS) might be useful as a bifidobacterial growth factor [16] or for attenuating the intestinal disorders of elderly patients [17]. Nevertheless, production of GOS by the TG reaction has recently become of interest. Glycoside moieties of glycoconjugates participate in recognition phenomena, and the synthesis of related structures can be performed using β-galactosidase [18]. The structure elucidation of TG products showed generally the prevailing formation of $\beta(1,6)$ linkages. Nevertheless, the synthesis reaction also can form other linkages, such as $\beta(1,4)$ galactosidic linkage [19].

In previous papers it was reported that *B. bifidum*, a bacterium from human colon, is able to exert a beneficial influence on the health of breast-fed infants [20–21]. We showed that a β-D-galactosidase from *B. bifidum* exhibited a transgalactosylase (TGase) activity [22]. The structure determination of ten GOS synthesized from lactose showed the predominance of $\beta(1,3)$ linkages; such GOS had never previously been shown to be products of TG. Hence, it was of interest to know whether *B. bifidum* was able to synthesize *in vivo* such GOS structures. The GOS production was optimized, and the properties of the enzyme are described and discussed. Previous experiments indicated the presence of different β-D-galactosidases in *B. bifidum* cells [23]. The purification of these enzymes allowed us to determine whether all or only one β-D-galactosidase was responsible for the specific TG reaction.

Experimental

Materials

BSA, *p*-nitrophenyl glycosides and chemicals were purchased from Sigma. Lactose, glucose and galactose were obtained from Merck. Hydroxyapatite Ultrogel, and Q Fast Flow, Mono P, Superose 6 and Polybuffer 74 were products of Sepracor and Pharmacia respectively. Glucose 6-phosphate dehydrogenase, hexokinase, NADP, ATP, and $MgSO_4$ were purchased from Boehringer Mannheim. Aminex HPX 42C columns (300 mm × 7.8 mm), Bio-Rad protein assay reagent and molecular-mass standards for SDS/PAGE were obtained from Bio-Rad, and molecular-mass standards for gel chromatography were from Pharmacia.

Organisms

Cultivation conditions for *B. bifidum* (DSM 20082) were optimized by Boutry [24]. Cultures were grown in anaerobic medium in a Setric fermenter with a final working volume of 200 litres at constant pH 6 and at 37 °C. Cells were harvested by centrifugation at 7 000 ***g***, 10 h after the end of the exponential phase of growth.

Protein assay

Protein was measured by the method of Bradford [25], with BSA as standard.

Enzyme assays

Hydrolytic activity was determined with 1.25 mM *p*-nitrophenyl galactoside (pNP-Gal) as substrate in 0.1 M sodium phosphate buffer, pH 6.5. When lactose was used as the substrate, the glucose was assayed by the method of Finch [26]. One unit of β-D-galactosidase was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol or 1 μmol of glucose/min at 37 °C and at pH 6.5.

TGase activity was measured by an h.p.l.c. method. The incubation mixture was composed of 100 μl of diluted enzyme, 300 μl of 0.05 M sodium citrate/0.1 M disodium phosphate buffer, pH 4.25, and 100 μl of 0.5 M lactose. After 4 h at 45 °C, the enzymic reaction was stopped by heating for 2 min on a boiling-water bath, then the incubation mixture was centrifuged for 30 min at 35 000 ***g***. The supernatant was diluted four times with water before injection (10 μl) on to an h.p.l.c. column (Aminex HPX 42C) thermostatically controlled at 85 °C in a SP 8100 apparatus (Thermo Separation Products). Oligosaccharides were eluted by de-aerated water at a flow rate of 0.75 ml/min and detected by refractometry. Oligosaccharide concentrations were calculated by using 10 μl of external standard (20 mM lactose) as reference. The results were expressed as trisaccharide equivalents. A unit of TGase was defined as the amount of enzyme catalysing the formation of 1 μmol of trisaccharide/min at 45 °C and at pH 4.25.

Determination of optimal conditions

The optimal temperature was determined according to the standard procedure at temperatures ranging from 4 to 60 °C. For the TG reaction, the enzyme was incubated with 0.24 M lactose in 0.05 M sodium citrate/0.1 M disodium phosphate, pH 4.8, for 20 h in the same range of temperature. Saccharides were separated by t.l.c. on Kieselgel 60 (Merck) in the solvent system butanol/water/acetic acid (2:1:1, v/v). Staining was done by spraying orcinol (0.2%) in 20% (v/v) H_2SO_4 and heating for 5 min at 120 °C. Saccharides were estimated by densitometry (Bio-Rad 620 densitometer).

The optimal pH for hydrolytic activity was determined using pNP-Gal as substrate in 0.1 M sodium citrate/phosphate/borate buffer, which had a pH range from 4 to 10. This buffer was chosen because it covers a wide range of pH without changing the nature of the salt. For the TG reaction, 50 μl of β-D-galactosidase were incubated with 0.24 M lactose at different pH values. After 20 h at 45 °C, the incubation was stopped by heating and the reaction products were estimated with TLC, as described above.

Influence of lactose concentration on GOS production

Cells were used for kinetic studies with various lactose concentrations (100, 250 or 400 mM) in 0.05 M sodium citrate/0.1 M disodium phosphate buffer, pH 4.25. Samples (0.4 ml) were withdrawn at various times from 30 min up to 48 h and the reactions stopped by heating. After centrifugation and dilution with water, the reaction products were analysed by h.p.l.c.

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