Expression and Characterization of Two β-Galactosidases from *Klebsiella pneumoniae* 285 in *Escherichia coli* and their Application in the Enzymatic Synthesis of Lactulose and 1-Lactulose

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Z. Naturforsch. **69c**, 479 – 487 (2014) / DOI: 10.5560/ZNC.2014-0061
Received March 16 / October 30, 2014 / published online December 22, 2014

The two genes *lacZ1* and *lacZ2* from *Klebsiella pneumoniae* 285, encoding β-galactosidase isoenzymes II and III (KpBGase-II and -III), were each cloned downstream of a T7 promoter for expression in *Escherichia coli* BL21(DE3), and the resulting recombinant enzymes were characterized in detail. The optimum temperature and pH value of KpBGase-II were 40 °C and 7.5, and those of KpBGase-III were 50 °C and 8.0, respectively. KpBGase-III was more stable than KpBGase-II at higher temperature (>60 °C). Both β-galactosidases were more active towards *o*-nitrophenyl-β-D-galactopyranoside as compared to lactose. The enzymatic synthesis of lactulose and 1-lactulose catalyzed by KpBGase-II and KpBGase-III was investigated. Using 400 g/L lactose and 200 g/L fructose as substrates, the resulting lactulose and 1-lactulose yields with KpBGase-II were 6.2 and 42.3 g/L, while those with KpBGase-III were 5.1 and 23.8 g/L, respectively. KpBGase-II has a potential for the production of 1-lactulose from lactose and fructose. Like other β-galactosidases, the two isozymes catalyze the transgalactosylation in the presence of fructose establishing the β-(1→1) linkage.

Key words: Klebsiella pneumoniae, β-Galactosidase, 1-Lactulose

Introduction

Transgalactosylation products of β-galactosidases such as galacto-oligosaccharides (GOS) and [β-D-galactosyl-(1→4)-fructose] lactulose have been proven effective prebiotic food ingredients beneficial to human health (Sako *et al*., 1999). Currently, it is generally accepted that the transgalactosylation reaction occurs with retention of the stereochemistry at the anomeric centre of galactose. In brief, a covalent bond forms between the galactosyl moiety and the enzyme, followed by the galactosyl transfer to a nucleophile acceptor (Gosling *et al*., 2010). The product of the β-galactosidase-catalyzed reaction depends on the type of the galactosyl acceptor. When it is lactose or fructose, the enzyme will catalyze the formation of GOS or lactulose, respectively. Besides lactulose, further three glycosidic molecules, namely, [β-D-galactosyl-(1→1)-fructose] 1-lactulose, 5-lactulose, and 6-lactulose, are formed from lactose during the enzymatic transgalactosylation in the presence of fructose. One of the most important regioisomers of lactulose is 1-lactulose. We have recently reviewed the production of 1-lactulose by β-galactosidases from *Escherichia coli*, *Aspergillus oryzae*, and *Kluyveromyces lactis* (Wang *et al*., 2013). The enzymatically produced 1-lactulose had a bifidogenic effect and may potentially be used as a prebiotic.

Concerning the enzymatic synthesis of lactulose and 1-lactulose, the most studied β-galactosidases are those from *K. lactis* and *A. oryzae* (Ajisaka

et al., 1988; Guerrero *et al*., 2011; Förster-Fromme *et al*., 2011; Song *et al*., 2012; Hua *et al*., 2012). Although *A. oryzae* β-galactosidase exhibits high transgalactosylation activity, lactulose synthesis by this β-galactosidase has not been exploited on a commercial scale to date. Therefore, an efficient enzymatic process still needs to be developed. Despite much effort concentrating on eukaryotes (yeasts and moulds) in the past few decades, screening for new strains producing β-galactosidase with high transgalactosylation activity from prokaryotic microorganisms, or generating the desired properties of β-galactosidases by directed evolution, will be of great interest for increasing lactulose and 1-lactulose yields.

In this context, a strain producing β-galactosidases was isolated from soil surrounding a factory dairy farm and designated *Klebsiella pneumoniae* 285 (Jiang *et al*., 2010). This strain contained three β-galactosidases, namely, a plasmid-encoded β-galactosidase (termed KpBGase-I), a chromosomal β-galactosidase (termed KpBGase-II, encoded by the *lacZ1* gene), and a third β-galactosidase (termed KpBGase-III, encoded by the *lacZ2* gene) (Hall and Reeve, 1977). To date, only the third β-galactosidase, KpBGase-III, has been characterized, particularly with respect to its kinetic properties and substrate specificity (Hall and Reeve, 1977; Hall, 1980). To the best of our knowledge, the utilization of these three β-galactosidases for lactulose and GOS synthesis has not been investigated. Therefore, the study of these enzymes will provide insight into their biochemical properties and functions that may differ from those of other β-galactosidases. In the present study, the two β-galactosidase genes, *lacZ1* and *lacZ2*, of *K. pneumoniae* 285 were expressed heterologously, and the biochemical properties of the corresponding recombinant proteins, *i. e.* KpBGase-II and KpBGase-III, were analysed. The applicability of the two enzymes to the synthesis of prebiotic lactulose and 1-lactulose from lactose and fructose was also investigated.

Materials and Methods

Materials

K. pneumoniae 285 had previously been isolated by Jiang *et al*. (2010). This strain has been deposited at the Culture and Information Centre of Industrial Microorganisms of China Universities (Wuxi, China) with the accession number CICIM B7001. PrimeSTAR® HS DNA polymerase was purchased from Takara (Dalian, China). *E. coli* DH5α was used for gene cloning. *E. coli* BL21(DE3) and pET-28a(+) were used as host and expression vectors, respectively. Lactose, fructose, *o*-nitrophenol (ONP), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG), all of analytical grade, were from Sinopharm Chemical Reagent (Shanghai, China) and used without further purification. All standards for high performance liquid chromatography (HPLC) analysis were purchased from Sigma-Aldrich (Shanghai, China). The standard of 1-lactulose (purity > 98 %) was prepared in our laboratory. Other chemicals used were of analytical grade and available commercially.

Gene cloning and plasmid construction

The gene *lacZ1* encoding KpBGase-II was amplified from the genomic DNA of *K. pneumoniae* 285 by the polymerase chain reaction (PCR), using the following primers: 5′-CGCGGATCCATGCAAATTAGCGATACC-3′ and 5′-CTCAAGCTTGAAGGCAACGCAAAAAGAACTC-3′, where the underlined bases represent *Bam*HI and *Hin*dIII restriction sites, respectively. The gene *lacZ2* encoding KpBGase-III was amplified by the same procedure using the primers: 5′-CGCCATATGAATAAATTTGCACCTTTAC-3′ and 5′-CTTAAGCTTTTAGGCGTGACGACGAAG-3′, where the underlined bases represent *Nde*I and *Hin*dIII restriction sites, respectively. PCR amplification was carried out using PrimeSTAR® HS DNA polymerase. The PCR products were then gel-purified, digested by *Bam*HI/*Hin*dIII and *Nde*I/*Hin*dIII restriction enzymes, respectively, and inserted into the corresponding sites of the pET-28a(+) expression vector, yielding pET-28a-*lacZ1* and pET-28a-*lacZ2*, respectively. The *lacZ1* and *lacZ2* genes were deposited in the GenBank database under accession numbers HQ324709 and HQ324708, respectively.

Expression and purification of recombinant KpBGase-II and KpBGase-III

E. coli BL21(DE3) cells were transformed with pET-28a-*lacZ1* and pET-28a-*lacZ2*, respectively. The engineered strains were grown in Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin at 37 °C until the OD_{600} had reached 0.4 to 0.6, at which point 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to induce gene expression. After induction at 30 °C for 10 h, the induced cells were harvested by centrifugation and

lysed using a Vibra Cell™ 72405 sonicator (Sonics and Material, Newtown, CT, USA). The soluble fraction was loaded onto an Ni^{2+} chelating Sepharose HP chromatography column (GE Healthcare Biosciences AB, Uppsala, Sweden). The His-tagged target protein was eluted with imidazole in a range of 100 – 250 mM. The purities of recombinant KpBGase-II and KpBGase-III were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay

Enzyme activity was assayed by measuring the rate of the hydrolysis of ONPG. In brief, an 800-μL reaction buffer containing 6.64 mM ONPG dissolved in 20 mM potassium phosphate buffer (pH 7.5) was incubated at the corresponding optimum temperature (40 °C for recombinant KpBGase-II and 50 °C for recombinant KpBGase-III). The reaction was initiated by adding 200 μL of appropriately diluted enzyme. After 5 min, the reaction was terminated by the addition of 1 mL of 10% (w/v) Na_2CO_3, and then the amount of the formed ONP was determined by its absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG per min under assay conditions (40 °C and pH 7.5 for KpBGase-II; 50 °C and pH 8.0 for KpBGase-III).

Characterization of recombinant KpBGase-II and KpBGase-III

The effects of the pH value on the activities of recombinant KpBGase-II and KpBGase-III (32.2 μg, 2 U, for KpBGase-II, and 53.9 μg, 2 U, for KpBGase-III) were determined at the optimal temperature of each enzyme (40 °C for KpBGase-II and 50 °C for KpBGase-III) within the pH range 5.0 – 9.0. The buffers used were 100 mM potassium hydrogen phosphate-citrate buffer (pH 5.0 to 7.0), 100 mM potassium phosphate buffer (pH 7.0 to 8.0), and 100 mM Tris-HCl buffer (pH 8.0 to 9.0). To determine the pH stability, samples were pre-incubated in the above-mentioned buffers for 2 h in an ice-bath, and the remaining activity was determined under standard assay conditions. The temperature profiles of recombinant KpBGase-II and KpBGase-III were measured by incubating each enzyme in a buffer of optimal pH value (7.5 for KpBGase-II and 8.0 for KpBGase-III) at temperatures ranging from 35 to 65 °C. Thermostabilities of recombinant KpBGase-II and KpBGase-III were monitored by incubating the enzyme solutions at different temperatures (40, 50, and 60 °C) in 100 mM potassium phosphate buffer (pH 7.5). An aliquot of the enzyme solution was withdrawn at each time interval, and the residual activity was measured under standard assay conditions. The effects of metal ions and EDTA on the activities of the enzymes were determined by measuring the activity under standard conditions after pre-incubation in 100 mM potassium phosphate buffer (pH 7.5) for 1 h at 37 °C in the presence of salts of the metal ions Na^+, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, and Zn^{2+}, and EDTA, each with a final concentration of 5 mM. The control reaction without addition of metal ion or EDTA was performed in parallel, and its activity was designated 100%. The K_m, V_{max}, and k_{cat} values for the enzymes were determined by analysing the initial velocities at different ONPG concentrations (1.0 – 8.33 mM). The Michaelis-Menten constants were obtained from Lineweaver-Burk plots.

Transgalactosylation reactions

A lactose solution of 400 g/L was prepared by dissolving lactose in 100 mM potassium phosphate buffer (pH 7.5). The transgalactosylation reaction was allowed to proceed in a final volume of 10 mL containing 400 g/L lactose and 200 g/L fructose at the optimal temperature of each enzyme (40 °C for KpBGase-II and 50 °C for KpBGase-III) for 8 h. The reaction was initiated by the addition of each β-galactosidase at a final enzyme concentration of 3.5 U/mL (corresponding to 56.4 μg for KpBGase-II and 94.4 μg for KpBGase-III). Samples (500 μL) were withdrawn at specific time intervals and immediately heated in boiling water for 10 min to inactivate the enzyme.

HPLC analysis

The sugars lactose, fructose, glucose, galactose, lactulose, and 1-lactulose in the assay solution were determined using an HPLC system (Hitachi L-2000; Hitachi, Tokyo, Japan) equipped with a refractive index (RI) detector and a high-performance sugar column (Shodex® Asahipak NH_2P-50 4E; Shodex, Tokyo, Japan). The column was kept at 30 °C and eluted with a mobile phase of acetonitrile/water (75:25, v/v) at a flow rate of 1.0 mL/min. The sugars were identified by comparison of their retention times with those of the corresponding standards.

Conversions of lactose and fructose

Percentage of conversion of lactose was expressed as:

$$\text{lactose conversion } (\%) = \frac{C_{IL} - C_{RL}}{C_{IL}} \cdot 100,$$

where C_{IL} and C_{RL} represent the initial concentration of lactose and its concentration remaining after the reaction, respectively.

Percentage of conversion of fructose was expressed correspondingly.

Results and Discussion

Expression and purification of recombinant KpBGase-II and KpBGase-III

Each of the genes *lacZ1* and lacZ2, encoding KpBGase-II and KpBGase-III, respectively, was subcloned into the pET-28a(+) vector and expressed as a His-tagged protein. The resulting expression vectors, pET-28a-*lacZ1* and pET-28a-*lacZ2*, were transformed into *E. coli* BL21(DE3) competent cells. Large-scale expression was carried out in 1-L flasks. The N-terminal 6× histidine-fused recombinant KpBGase-II and KpBGase-III proteins were purified from the cell lysates by Ni^{2+} affinity chromatography and then subjected to SDS-PAGE analysis. The SDS-PAGE separation of the purified KpBGase-II and KpBGase-III revealed distinct protein bands of different sizes. As shown in Fig. 1a, a strong band with an apparent molecular weight of 116 kDa was observed for the purified KpBGase-II preparation (lane 2), which corresponded well to the theoretical molecular mass (117.6 kDa) of KpBGase-II. On the other hand, a less abundant band of an apparent mass slightly higher than 66.4 kDa in KpBGase-III corresponded to the theoretical value (77.4 kDa) of KpBGase-III (Fig. 1b, lane 1). Thus, the two recombinant enzymes were expressed in *E. coli* BL21(DE3).

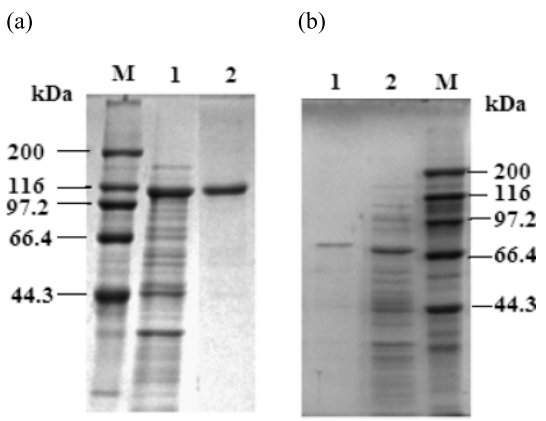

Fig. 1. SDS-PAGE analysis of the purified recombinant KpBGase-II and KpBGase-III. (a) Lane M, molecular mass standard proteins; lane 1, cell lysate from *E. coli* BL21(DE3) harbouring pET-28a-*lacZ1*; lane 2, purified KpBGase-II obtained after nickel column affinity chromatography. (b) Lane M, molecular mass standard proteins; lane 1, purified KpBGase-III obtained after nickel column affinity chromatography; lane 2, cell lysate from *E. coli* BL21(DE3) harbouring pET-28a-*lacZ2*.

Characterization of recombinant KpBGase-II and KpBGase-III

Effects of pH value and temperature

As shown in Fig. 2, the optimal pH value for the activity of recombinant KpBGase-II was 7.5 (Fig. 2a), while it was 8.0 for KpBGase-III (Fig. 2b). Moreover, recombinant KpBGase-II and KpBGase-III were active over the pH range of 5.0 – 9.0. In potassium phosphate buffer (pH 7.0 or pH 8.0), the activities of both enzymes were higher than those in potassium hydrogen phosphate-citrate buffer (pH 7.0) or Tris-HCl buffer (pH 8.0) at the same pH. When tested for pH stability (pH 5.0 – 9.0), both enzymes proved to be stable in the alkaline pH range (Figs. 2a and 2b).

KpBGase-II and KpBGase-III exhibited optimal activity at 40 and 50 °C, respectively (Fig. 2c). Moreover, the activity of KpBGase-II gradually decreased at temperatures higher than 40 °C, and was almost completely lost at 60 °C, whereas the activity of KpBGase-III decreased rapidly in the range of 55 and 60 °C. As seen in Fig. 2, KpBGase-II (Fig. 2d) and KpBGase-III (Fig. 2e) exhibited comparably good thermostabilitiy at 40 and 50 °C.

Effects of metal ions and EDTA

It has been reported that monovalent or divalent cations affect the activity of β-galactosidases (Juers *et al.*, 2009). To test a cation effect on the activities of the two KpBGases, the enzymes were pre-incubated in the presence of 5 mM salts or EDTA for 1 h at 37 °C prior to the addition of ONPG, and the residual activity was calculated relative to that of the control (Table I).

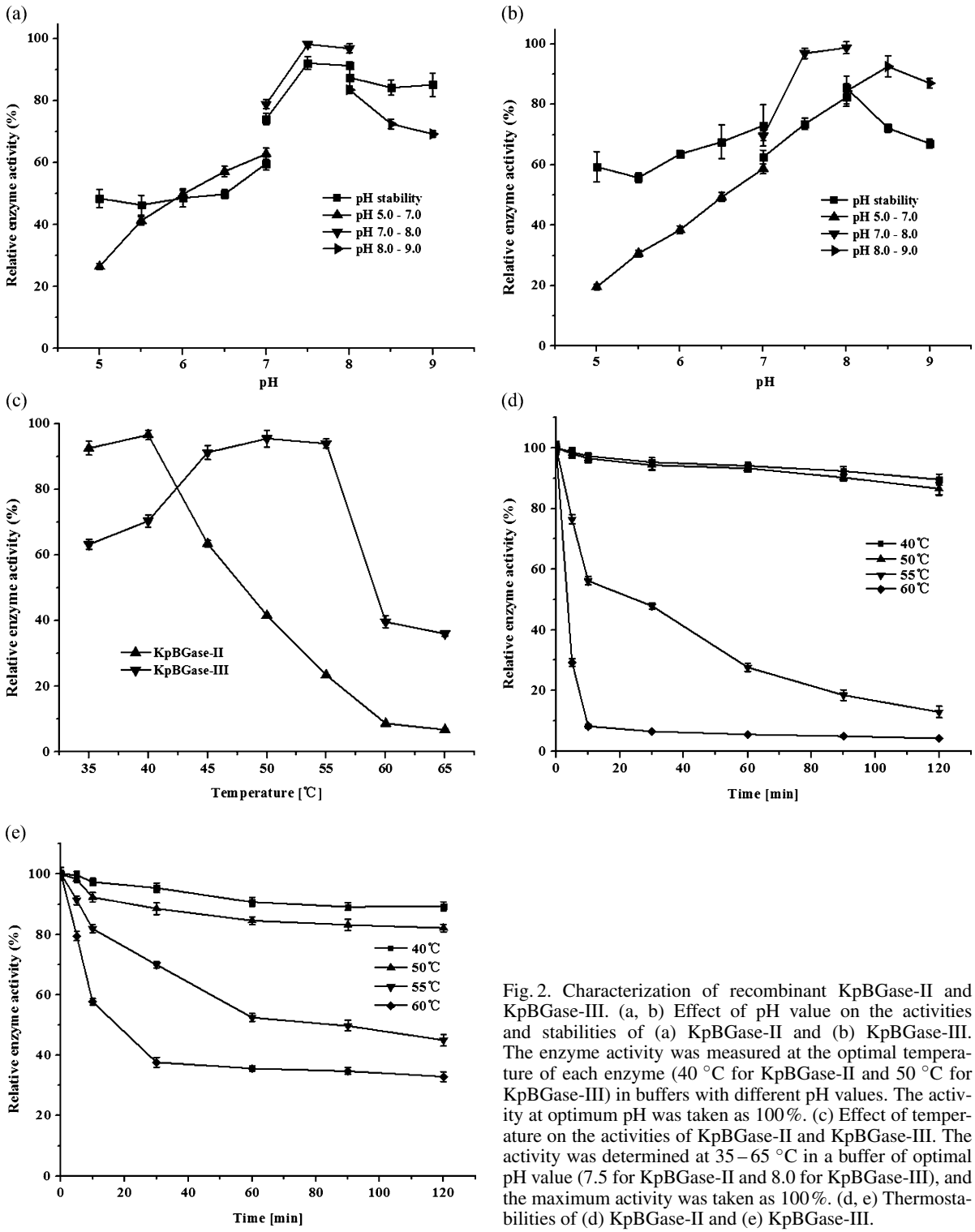

Fig. 2. Characterization of recombinant KpBGase-II and KpBGase-III. (a, b) Effect of pH value on the activities and stabilities of (a) KpBGase-II and (b) KpBGase-III. The enzyme activity was measured at the optimal temperature of each enzyme (40 °C for KpBGase-II and 50 °C for KpBGase-III) in buffers with different pH values. The activity at optimum pH was taken as 100%. (c) Effect of temperature on the activities of KpBGase-II and KpBGase-III. The activity was determined at 35 – 65 °C in a buffer of optimal pH value (7.5 for KpBGase-II and 8.0 for KpBGase-III), and the maximum activity was taken as 100%. (d, e) Thermostabilities of (d) KpBGase-II and (e) KpBGase-III.

Table I. Effects of 5 mM metal ions and EDTA on the activities of recombinant KpBGase-II and KpBGase-III.

Metal ion or EDTA	Relative activity (%)	
	KpBGase-II	KpBGase-III
None	100 ± 1.5	100 ± 1.2
Na^{+}	98.5 ± 1.4	119.7 ± 2.62
Fe^{2+}	140.3 ± 2.8	97.9 ± 1.9
Mg^{2+}	156.8 ± 3.3	121.4 ± 1.1
Ca^{2+}	99.0 ± 1.7	98.8 ± 2.1
Cu^{2+}	26.4 ± 1.3	9.5 ± 0.6
Mn^{2+}	157.2 ± 1.8	133.5 ± 2.5
Co^{2+}	132.7 ± 1.9	98.6 ± 1.0
Pb^{2+}	79.0 ± 0.8	99.3 ± 3.0
Zn^{2+}	138.1 ± 0.6	99.8 ± 1.2
EDTA	27.7 ± 0.8	22.5 ± 0.5

Cu^{2+} caused a significant loss of the activities of both enzymes, while Mg^{2+} and Mn^{2+} had a positive effect. Na^{+} and Ca^{2+} had no effect on the activity of KpBGase-II, and Fe^{2+}, Co^{2+}, and Zn^{2+} were stimulatory. Fe^{2+}, Ca^{2+}, Co^{2+}, Pb^{2+}, and Zn^{2+} did not affect the activity of KpBGase-III, and Na^{+} had a small stimulatory effect. However, the activity of KpBGase-II was decreased by 21 % in the presence of Pb^{2+}. EDTA inhibited the activities of the two enzymes, suggesting that the divalent metal ions Mg^{2+} and/or Mn^{2+} are required for the activities of both KpBGases.

Kinetic analysis

The K_m, V_{max} and k_{cat} values for ONPG were 0.72 mM, 58.8 μmol/[min mg protein], 121 s^{-1} for KpBGase-II, and 0.82 mM, 227.3 μmol/[min mg protein], 48 s^{-1} for KpBGase-III, respectively.

Enzymatic synthesis of lactulose and 1-lactulose catalyzed by recombinant KpBGase-II and KpBGase-III

The reaction was performed at the respective optimum temperature, *i. e.* 40 °C for KpBGase-II and 50 °C for KpBGase-III, and 400 g/L lactose. After incubation for 6 h, the reaction products were analysed by HPLC. Figure 3 shows a typical chromatogram of the reaction products. Under the conditions described, lactose was not completely hydrolyzed by either KpBGase-II or KpBGase-III to galactose and glucose within 6 h. Moreover, the residual lactose concentration in the incubation mixture with KpBGase-III was much higher than with KpBGase-II, indicating that KpBGase-II hydrolyzes lactose more actively than KpBGase-III. In addition to lactose hydrolysis, two galacto-oligosaccharides, GOS-1 and GOS-2 (unidentified oligosaccharides with retention times of ca. 21.7 min and ca. 30.4 min, respectively), were produced (Fig. 3), their amounts being somewhat higher in the case of KpBGase-II. According to our results, both KpBGase-II and KpBGase-III had a high transgalactosylation and a weak hydrolytic activity, respectively. Thus, the two recombinant enzymes were employed as catalysts for the enzymatic synthesis of lactulose and 1-lactulose.

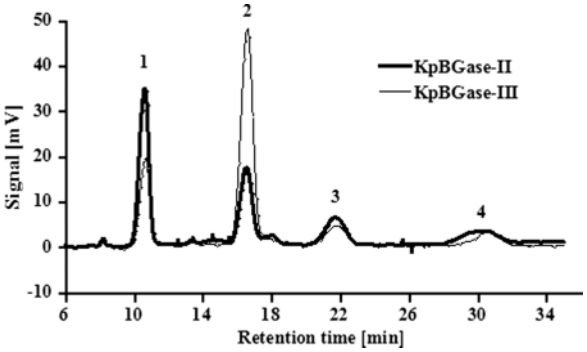

Fig. 3. HPLC profiles of the bioconversion of 400 g/L lactose catalyzed by recombinant KpBGase-II and KpBGase-III at the respective optimum temperature, *i. e.* 40 °C for KpBGase-II and 50 °C for KpBGase-III, for 6 h. 1, Glucose and galactose; 2, lactose; 3, GOS-1; 4, GOS-2.

For lactulose and 1-lactulose production, 10 mL of 400 g/L lactose and 200 g/L fructose were incubated with 3.5 U/mL of KpBGase-II and KpBGase-III, respectively. Samples were withdrawn every hour for up to 8 h and their sugar composition analysed. With KpBGase-II (Fig. 4a), the 1-lactulose yield reached a plateau at 7 h, and a similar behaviour was observed for KpBGase-III, with a plateau reached at 6 h (Fig. 4b). The 1-lactulose yields obtained with KpBGase-II and KpBGase-III were 42.3 g/L and 23.8 g/L (Fig. 4), respectively, indicating that KpBGase-II was more effective in the enzymatic synthesis of 1-lactulose than KpBGase-III.

A comparison of lactulose and 1-lactulose production from lactose and fructose by β-galactosidases from various sources is summarized in Table II. For all enzymes, the yield of 1-lactulose was consistently higher than that of lactulose, suggesting that the production of 1-lactulose is independent of the enzyme source. Ajisaka *et al.* (1988) reported that the relative yields of lactulose and 1-lactulose depended on incubation conditions such as pH value, time, oper-

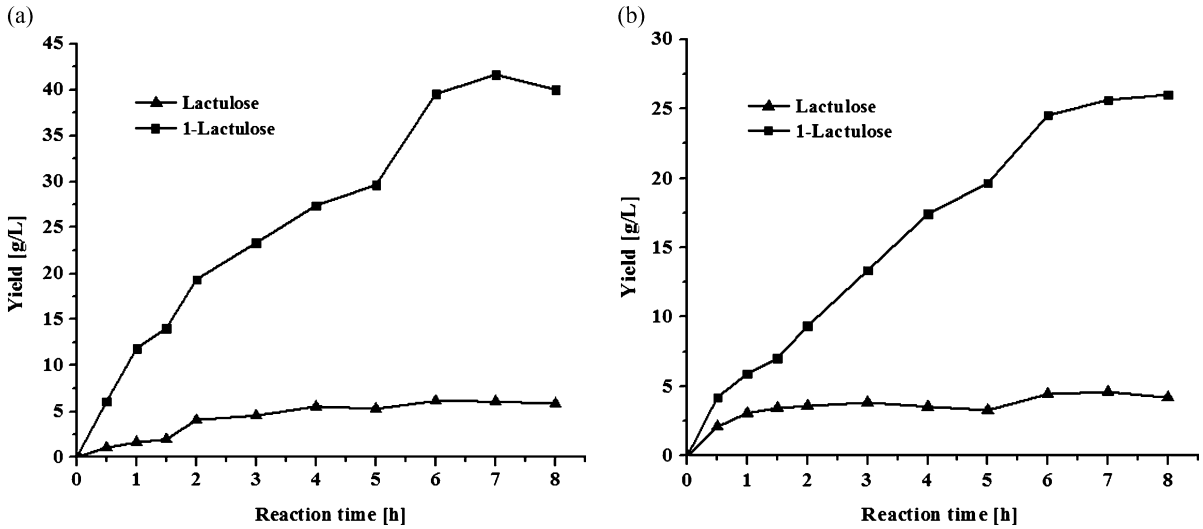

Fig. 4. Time course of the transgalactosylation reaction producing lactulose and 1-lactulose from 400 g/L lactose and 200 g/L fructose. The reaction was carried out at the optimal pH value and temperature of the respective enzyme (40 °C and pH 7.5 for KpBGase-II; 50 °C and pH 8.0 for KpBGase-III). (a) KpBGase-II; (b) KpBGase-III.

Table II. Comparison of lactulose and 1-lactulose production by recombinant KpBGase-II and KpBGase-III and other β-galactosidases.

Enzyme source	Initial lactose concentration [g/L] (initial fructose concentration [g/L])	Lactose conversion (%)	Fructose conversion (%)	Lactulose yield [g/L]	1-Lactulose yield [g/L]	Reference
KpBGase-II	400 (200)	86.7	35.5	6.2	42.3	This study
KpBGase-III	400 (200)	57.5	36.5	5.1	23.8	This study
Escherichia coli[a]	(500)			3.3	8	Ajisaka *et al.* (1987)
Escherichia coli[b]	(1000)			10.5	33.6	Ajisaka *et al.* (1988)
Aspergillus oryzae[c]	(1000)			10.4	32.8	Ajisaka *et al.* (1988)
Aspergillus oryzae	40 (90)	66.0		3.05	4.03	Förster-Fromme *et al.* (2011)
Maxilact® 5000[d]	200 (200)	[d] 66.5		15.4	47.5	Shen *et al.* (2012)
Maxilact® 5000[d]	250 (100)	87.1	33.4	8	22	Hua *et al.* (2012)

[a] Transgalactosylation reaction was performed in 0.1 M potassium phosphate buffer solution (pH 6.8) containing 100 g/L galactose and 500 g/L fructose, and 0.19 U/mg β-galactosidase from *E. coli* immobilized on Eupergit C.
[b] Transgalactosylation reaction was performed in 0.1 M sodium phosphate buffer solution (pH 7.3) containing 100 g/L galactose and 1000 g/L fructose, and 580 U/mg β-galactosidase from *E. coli* immobilized on Eupergit C.
[c] Transgalactosylation reaction was performed in 0.1 M sodium phosphate buffer solution (pH 7.3) containing 100 g/L galactose and 1000 g/L fructose, and 107 U/mg β-galactosidase from *A. oryzae* immobilized on Eupergit C.
[d] Maxilact® 5000 is a β-galactosidase commercially produced by *K. lactis*.

ation mode (batch or continuous incubation, respectively), and substrate ratio. Interestingly, the yield of 1-lactulose was much higher than that of any of the three other lactulose regioisomers, *viz.* lactulose, 5-lactulose, and 6-lactulose (Ajisaka *et al.*, 1988). Taken together, the fact that 1-lactulose was the main transgalactosylation product of all tested β-galactosidases suggests that the active sites of the β-galactosidases of *E. coli*, *A. oryzae*, *K. lactis* or *K. pneumoniae* responsible for the transgalactosylation have been conserved during evolution. Shen *et al.* (2012) observed that the 1-lactulose yield obtained with Maxilact® 5000 was not simply correlated with the rate of lactose conversion, while in our experiments increased lactose conversion did lead to an increased 1-lactulose yield (Table II). Based on these data, KpBGase-II can be considered a suitable biocatalyst for the enzymatic synthesis of 1-lactulose.

As 1-lactulose has a bifidogenic effect (Förster-Fromme *et al*., 2011), the large-scale production of 1-lactulose by β-galactosidases with high transgalactosylation activity has spurred research activities from lab-scale (Ajisaka *et al*., 1988; Shen *et al*., 2012; Hua *et al*., 2012) to pilot plant-scale, up to a volume of 170 L (Förster-Fromme *et al*., 2011). Immobilization, combined with packed-bed reactor operation, will be more suitable and efficient for the production of 1-lactulose by β-galactosidase.

Brás *et al*. (2010) concluded that the *E. coli* β-galactosidase has a transgalactosylation preference for glycosidic bonds in the order β-(1→6) > β-(1→4) > β-(1→3) on the basis of theoretical calculations of the catalytic mechanism. Therefore, these authors postulated that allolactose, *i. e.* β-D-galactosyl-(1→6)-glucose, is the preferred product when using lactose as the natural substrate, and this was confirmed by their experimental data (Brás *et al*. 2010). However, when β-galactosidase from the same source was employed to produce lactulose, the β-(1→1) glycosidic bond was preferentially formed in the transgalactosylation reaction, followed by the β-(1→4), β-(1→5), and β-(1→6) bond (Ajisaka *et al*., 1988). This difference in the transgalactosylation preference for the glycosidic bond β-(1→1) is likely due to mechanistic differences between β-galactosidases from different sources.

Conclusion

Recombinant KpBGase-II is a suitable biocatalyst for the production of 1-lactulose from lactose and fructose. Like other β-galactosidases of different origins, KpBGase-II and KpBGase-III have a transgalactosylation preference for establishing the β-(1→1) linkage during lactose cleavage in the presence of fructose. The differences in the transgalactosylation activities of KpBGase-II and KpBGase-III can presently not be explained. Further studies are needed to understand the mechanism of the transgalactosylation reaction and to improve the desired enzyme function through directed evolution of the respective two β-galactosidase genes.

Acknowledgement

This study was financially supported by the National Key Technology R & D Program in the 12th Five Year Plan of China (2011BAD23B03), the Key Project of National Natural Science Fund (31230057), and the PhD Program Foundation of Jiangnan University (JUDCF09025).

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