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A study of commercial β-galactosidase stability under simulated *in vitro* gastric conditions

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Abstract. β-Galactosidase activity in milk may be affected by several factors, such as temperature, pH, milk composition, and metal ions. It is important to note that digestive proteases and gastrointestinal pH can affect enzyme activity during transit through the gastrointestinal tract. For the investigation of commercial β-galactosidase stability in human and animal gastric tracts, human gastrointestinal tract (GIT) models were employed, enabling prediction of enzyme activity under *in vivo* conditions. The aim of this study was to analyse and compare commercial β-galactosidase stability under simulated *in vitro* gastric conditions. Commercial enzymes (Ha Lactase 5200 produced by *Kluyveromyces lactis* and NOLA™Fit5500 produced by *Bifidobacterium bifidum* expressed in *Bacillus licheniformis*, Chr. Hansen, Hørsholm, Denmark; GODO-YNL2 produced by *Kluyveromyces lactis*, Danisco, Copenhagen, Denmark) were used for this study. Commercial enzymes were added to GIT models at 1 and 5 mL L^{-1}. The enzyme activity was assessed as the percentage of lactose hydrolysis by the enzymes from *Kluyveromyces lactis* and *Bacillus licheniformis* using HPLC after digestion. β-Galactosidase extracted from yeast (*Kluyveromyces lactis*) and bacteria (*Bacillus licheniformis*) was found to be effective as a strategy for improving lactose tolerance.

Key words: β-galactosidase, simulated gastric conditions, lactose hydrolysis.

INTRODUCTION

Lactose is the major disaccharide in milk and can be hydrolysed into glucose and galactose by β-galactosidase (Gambelli, 2017). Lactose is digested *in vivo* by a membrane-bound enzyme of the small intestinal epithelial cells in the human body (O'Connell & Walsh, 2010). Lactose is hydrolysed most slowly out of all of the dietary sugars. The hydrolysis of lactose occurs at only half the rate of sucrose hydrolysis. Generally, carbohydrates increase intestinal calcium absorption, and lactose, which is predominantly present in dairy products, is the most effective calcium absorbing carbohydrate (Pérez et al., 2008). Consumption of lactose-containing dairy products should be minimised for individuals with lactose intolerance (Dutra Rosolen et al., 2015). Certain ethnic and racial groups have increased propensity for β-galactosidase deficiency in the digestive tract. Most Asians (more than 90%), Africans (80–100%), Native Americans (more than 90%) and Southern Europeans (more than 80%) are

reported to be lactose intolerant (Mlichová & Rosenberg, 2006), mainly due to a low level of β-galactosidase in the intestinal walls.

One potential solution to the lack of this enzyme is a strategy of oral consumption of β-galactosidase for deficient individuals whilst consuming products containing lactose (Bosso et al., 2015). The market offers a great variety of caplets, chewable tablets, or soft gel capsules which contain β-galactosidase that improve gastrointestinal digestion of lactose and eliminate the symptoms caused by β-galactosidase deficiency. The ability of β-galactosidase preparations in the gastrointestinal tract depends upon degradation and enzyme activity under physiological conditions (Wang et al., 2009). There are several factors which can affect activity of β-galactosidase, such as temperature, pH, gastric enzymes, and bile acids. Furthermore, depending upon the source of β-galactosidase extraction, the enzyme may display diverse properties in a variety of applications (Bosso et al., 2016). Enzymes which need to be released in the intestine, such as β-galactosidase, necessarily have to pass intact through the stomach and need to be resistant to acidic pH in the range of 1.0 to 3.0 (Bosso et al., 2015). Therefore, most supplemental β-galactosidase preparations are coated to prevent gastric inactivation, and become activated in the intestine (Wang et al., 2009).

Bosso et al. (2015) have shown that *A. oryzae* and *K. lactis* β-galactosidases are inactivated under simulated gastrointestinal digestive conditions. The enzyme from *A. oryzae* was less effective for lactose hydrolysis than the enzyme from *K. lactis.* These authors determined that lactose hydrolysis was over 90% with the highest concentration of the enzyme from *K. lactis* at 40 °C. The highest levels of hydrolysis were at 37 °C and at enzyme concentrations of 3 and 5 mL L^{-1} (Bosso et al., 2015). The principal objective of this study was to analyse and compare commercial β-galactosidase stability under simulated *in vitro* gastric condition at different concentrations.

MATERIALS AND METHODS

B-Galactosidase resistance to inactivation was evaluated under simulated human stomach and small intestine digestive conditions.

Chemicals

All chemicals KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, NaOH, HCl, $CaCl_2$, bile salt, porcine pepsin (EC 3.4.23.1), porcine trypsin (EC 3.4.21.4), bovine chymotrypsin (EC 3.4.21.1), porcine pancreatic α-amylase (EC 3.2.1.1), porcine pancreatic lipase (EC 3.1.1.3), porcine pancreatic colipase and D-lactose monohydrate, D-(+)-glucose, D-(+)-galactose (≥ 98%, HPLC) were purchased from Sigma-Aldrich (Riga, Latvia).

Commercial enzymes

Three commercial β-galactosidase preparations were used in this study: NOLA™Fit5500 and Ha-Lactase 5200 (Chr. Hansen, Hørsholm, Denmark) and GODO YNL2 (Danisco, Copenhagen, Denmark). NOLA™Fit5500 is a *Bifidobacterium bifidum* ß-galactosidase (lactase) produced by submerged fermentation on a vegetable substrate using a selected strain of *Bacillus licheniformis* kept under contained conditions and not present in the final product (NOLA™Fit5500 Product Information, 2017). The other two enzymes are of *Kluyveromyces lactis* origin, GODO-YNL2 and

Ha-Lactase 5200 are active at neutral condition pH 6.5–8.0 (Ha-Lactase 5200 Product Information, 2014). NOLA™Fit5500 enzyme is active under acidic conditions (optimum pH 5.0–7.0) according to the manufacturers' data. GODO-YNL2 and Ha-Lactase 5200 enzymes have an activity of 5,000 NLU (neutral lactase units) mL^{-1} and 5,200 NLU mL^{-1}, respectively, and NOLA™Fit5500 has an activity of 5,500 BLU (bifido lactase units) mL^{-1}. All enzymes remained fully active throughout the study.

***In vitro* digestion of commercial β-galactosidase**

Three experiments were carried out to determine the effect of simulated gastrointestinal tract conditions on commercial GODO-YNL2 and Ha-Lactase 5200 (*Kluyveromyces lactis*) and NOLA™Fit5500 (*Bacillus licheniformis*). The modified method of Minekus et al. (2014) for gastrointestinal incubation of β-galactosidase was used in order to determine enzyme activity. Concentrations of gastric (SGF) and intestinal (SIF) electrolyte stock solutions, as well as gastrointestinal enzyme activities (U ml^{-1} digesta) were calculated according to the international consensus reported by Minekus et al. (2014).

In all experiments, the enzyme concentrations were 1 and 5 mL L^{-1}, calculated as 5,000 and 25,000 NLU mL^{-1} for GODO-YNL2 enzyme, 5,200 and 26,000 NLU mL^{-1} for Ha-Lactase 5200 enzyme, and 5,500 and 27,500 BLU mL^{-1} for NOLA™Fit5500 enzyme (Fig. 1).

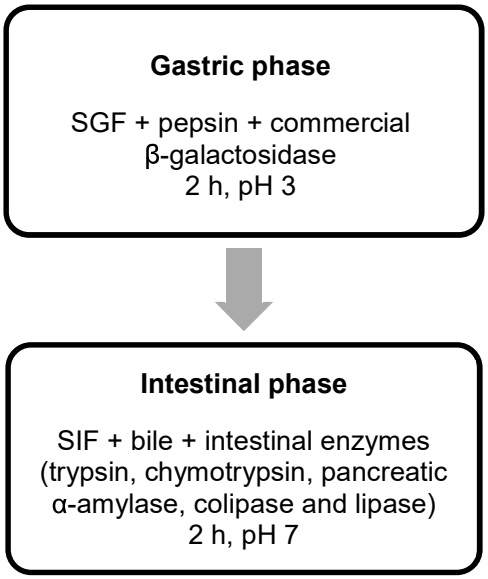

Figure 1. Diagram of a simulated *in vitro* digestion system. The GIT system (static gastrointestinal tract simulator) comprised of a single stirred tank reactor (Labfors 5, INFORS HT, Bottmingen, Switzerland) connected to the computer and controlled by bioprocess monitoring and control software 'Iris 6' (INFORS HT).

Each phase concentration/dosage of stock solution and enzyme activity were prepared according to Minekus et al. (2014).

Gastric phase

Liquid commercial β-galactosidase enzyme samples were mixed with 120 mL of simulated gastric fluid (SGF), which is the medium simulating gastric conditions in the fasted state, water, pepsin and 1 M HCl was added to reduce the pH to 3.0. Solution was incubated at 37 °C for 2 hours.

Intestinal phase

After 2 hours of gastric digestion, 160 mL of simulated intestinal fluid (SIF), intestinal enzymes and bile salt were added. NaOH (1 M) was used to raise the pH to 7.0 and the solution was incubated at 37 °C for another 2 hours. At this stage, 5% lactose solution was added to the flasks.

Sample preparation after digestion

The digested samples were taken after the small intestine digestion at the end of experiment, and β-galactosidase activity was measured. The samples were heated at 90–95 °C to inactivate the enzymes and kept at 4–6 °C until further analysis. The experiments were carried out with three replicates for each test.

Determination of lactose, glucose and galactose

The enzyme activity was determined according to percentage of lactose hydrolysis by the enzymes from *Kluyveromyces lactis* and *Bifidobacterium bifidum* using HPLC (Prominence HPLC system, Shimadzu LC-20, Torrance, CA, USA), refractive index detector RID-10A; Alltech NH_2, 4.6 mm x 250 mm, 5 µm column; temperature of 30 °C; mobile phase isocratic elution: A – acetonitrile 84%, B – deionized water 16%; capacity of the injection sample: 10 µL; total analysis time of up to 25 min; rate of flow: 1.0 mL min^{-1}.

Statistical analysis

Statistical analyses were performed with analysis of variance ANOVA and *Tukey* test at the significance level $P < 0.05$.

RESULTS AND DISCUSSION

All enzymes at a concentration of 1 mL L^{-1} showed higher lactose hydrolysis compared with 5 mL L^{-1} after simulation of enzyme passage through the stomach and small intestines (Table 1).

Table 1. Lactose hydrolysis (%) by *Kluyveromyces lactis* and *Bacillus licheniformis* β-galactosidases at different concentrations after intestinal phase digestion

Commercial enzyme	Hydrolysis, %	
	1 mL L^{-1}	5 mL L^{-1}
GODO-YNL2 *	42.5 ± 2.5 [a]	39.8 ± 4.8 [a]
NOLA™Fit5500 **	57.3 ± 4.3 [b]	49.1 ± 5.7 [b]
Ha-Lactase 5200 *	62.5 ± 3.9 [b]	56.7 ± 4.1 [c]

*Enzyme from *Kluyveromyces lactis;* **Enzyme from *Bacillus licheniformis.*
Results indicated with the same letter within a column do not differ significantly ($P < 0.05$).

Results showed that there is no significant difference between commercial enzyme concentrations ($F = 11.8$, $P > 0.05$), but in turn a significant difference between commercial enzyme preparates within each concentration was noticed ($F = 44.5$, $P < 0.05$).

Lactose hydrolysis was observed for all enzymes, however Ha-Lactase 5200 β-galactosidase showed greater hydrolysis for all concentrations studied. Contrary to yeast neutral lactase, acid lactase continues to hydrolyse lactose to approximately pH 4.5 and remains active in the presence of digestive enzymes and bile acids (Selvarajan & Mohanasrinivasan, 2015). This result can be explained by the findings of Vrese et al. (2001) where the effect was substantiated, largely due to digestion of lactose by the probiotic lactase activity, effectively performing the functions of a defective human enzyme, and also due to a slower transit time of the product.

Kwak (2001) evaluated the enzyme activity of microencapsulated β-galactosidase from *K. lactis* in a simulated human intestinal system (pH 7–8) and found 60.8 to 68.8% hydrolysis after 60 min of testing. The percentages are higher than the results found in the present study, where lactose hydrolysis up to 42.5% was obtained for GODO-YNL2 *K. lactis* β-galactosidase at 37 °C. However, lactose hydrolysis up to 62.5% was obtained for Ha-Lactase 5200 *K. lactis* β-galactosidase indicating a stronger resistance to digestion and higher activity of this enzyme. Lower lactose hydrolysis rates can be explained by the temperature. A temperature of 37 °C used in the GIT is lower than the optimal temperature of GODO-YNL2 enzyme (40 °C), but for NOLA™Fit5500 (35–50 °C) and Ha-Lactase 5200 (35–45 °C), is within the optimal range. Furthermore, Vidya et al. (2014) pointed out that the enzyme activity can be affected by the type of strain, cultivation conditions (temperature, pH, aeration, agitation and incubation time) and the growth media composition (particularly carbon and nitrogen sources). This leads to the conclusion that commercial β-galactosidase preparations produced from the same species, but using a different method which includes cultivation conditions and the growth medium composition, can impact enzyme physical properties.

Kotz et al. (1994) analysed β-galactosidase activity in conventional yoghurt and high lactase (HL) yoghurt during 60 min incubation at 37 °C and pH 3.5. The β-galactosidase in HL yoghurt was much less acid resistant than was the β-galactosidase in conventional yoghurt, likely due to the deactivation of β-galactosidase in the human gastrointestinal system. The authors assumed that β-galactosidase at high concentrations and at low pH is more sensitive to denaturation, and the dose of the enzyme is one of the factors which can impact degree of hydrolysis.

The results show that the highest degree of hydrolysis can be obtained using a β–galactosidase concentration of 1 mL L^{-1} (Fig. 2), rather than 5 mL L^{-1} (Fig. 3). The method used in this study was based on the proportions between the intake of food, digestive enzymes, and stock solution. This indicates that low concentrations of enzymes have a greater chance of maintaining activity by dissolving in a fermentable environment.

Metal ions can affect enzyme activity. For the yeast β-galactosidase which is isolated from *Kluyveromyces lactis* K^+ and Mg^{2+} worked as activators, whereas Ca^{2+} and Na^+ worked as inhibitors; for β-galactosidase from *Bacillus licheniformis* Ca^{2+}, Mn^{2+}, and Mg^{2+} were activators, while Cu^{2+}, Zn^{2+}, and Fe^{2+} were inhibitors (Zolnere, Liepins, & Ciprovica, 2017). For preparation of stock solutions according to Minekus (2014) a variety of salts were added (KCl; KH_2PO_4; $NaHCO_3$; NaCl; $MgCl_2(H_2O)_6$; $(NH_4)_2CO_3$;

$CaCl_2$) in different concentrations. This can also impact the amount of hydrolysed lactose (Adalberto et al., 2010).

Studying kinetic parameters of β-galactosidase from *Kluyveromyces lactis* Mateo et al., 2004 found that galactose was competitive and glucose noncompetitive inhibitor. Juajun et al. 2011 analysed bioconversion of lactose using β-galactosidase from *Bacillus licheniformis*, and the results showed that mainly D-galactose is an inhibitor.

Transgalactosylation is the reaction by which the enzyme β-galactosidase hydrolyzes lactose and transfers galactose to another carbohydrate, forming galactooligosaccharides (Otieno, 2010). Such reactions may explain why glucose and galactose concentrations are not equal upon lactose hydrolysis (Figs 2, 3).

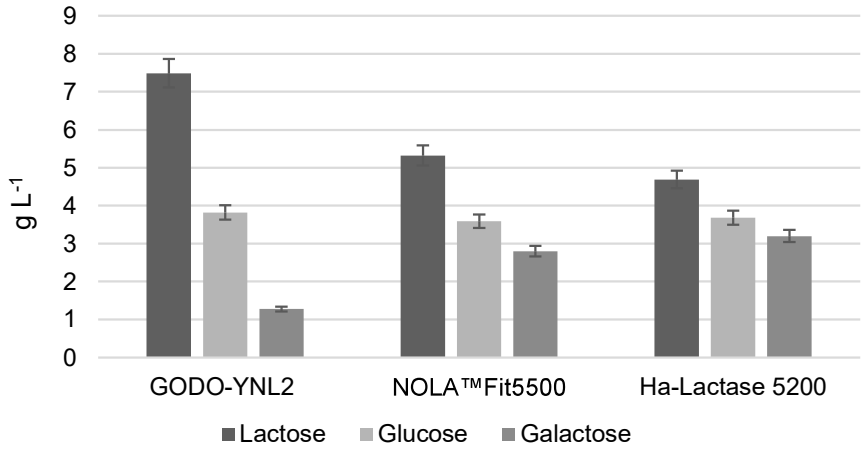

Figure 2. Sugars remaining after hydrolysis of an initial amount of 12.50 g in the GIT by *Kluyveromyces lactis* and *Bacillus licheniformis* commercial β-galactosidases at a concentration of 1 mL L^{-1}.

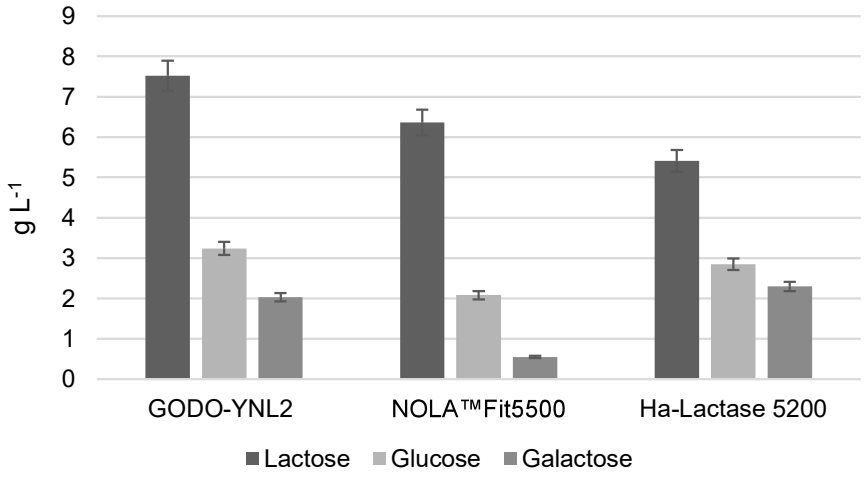

Figure 3. Sugars remaining after hydrolysis of an initial amount of 12.50 g in the GIT by *Kluyveromyces lactis* and *Bacillus licheniformis* commercial β-galactosidases at a concentration of 5 mL L^{-1}.

There were no statistical differences ($P < 0.05$) in lactose hydrolysis by enzymes from *B. licheniformis* and *K. lactis* under simulated intestinal conditions. Relatively high levels of lactose hydrolysis were obtained, especially for enzyme concentrations of 1 mL L^{-1}.

CONCLUSIONS

The present study indicates that β-galactosidases extracted from yeast (*Kluyveromyces lactis*) and bacteria (*Bacillus licheniformis*) were effective under *in vitro* digestive conditions as a strategy for improving lactose tolerance. The dose of β galactosidase enzyme is one of the factors which can impact upon the degree of lactose hydrolysis in the human gastrointestinal system. Enzymes at high concentrations and low pH are more sensitive to denaturation. Under *in vitro* conditions, the highest hydrolysis percentages were for NOLA™Fit5500 and Ha-Lactase 5200 enzymes, thus these were most effective at mitigating against lactose intolerance. In future, an encapsulation method may allow the creation of orally used β-galactosidase preparations to be consumed with food products containing lactose.

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