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THE IMPACT OF CALCIUM IONS ON COMMERCIALLY AVAILABLE β-GALACTOSIDASE

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Abstract

Commercial lactose hydrolysing enzymes producers provide purchasers with information as optimal enzyme temperature and pH, as well recommended amount of enzyme. However, there are also other indices in food substrate which might have impact on enzyme activity. The cations concentration in the substrate is the one of those. Whey is a dairy by-product with a relatively high Ca^{2+}, K^{+} and Na^{+} ions concentration. These cations (especially Ca^{2+}) are added in cheese making and remain in whey in comparatively large amounts. Moreover, Na^{+} also remains in whey since salt is added for cheese grains salting. The aim of the study is to determine the impact of Ca^{2+} ions on commercially available β-galactosidase. The effect of Ca^{2+} ions on commercial β-galactosidase (NOLA Fit5500, Ha-Lactase 5200, Chr. HANSEN, Denmark; Lactozym® Pure 6500 L, Novozymes, Denmark) were tested. For investigation of the effect of Ca^{2+} ions on the β-galactosidase activity, chromogenic lactose substare – oNPG (2-nitrophenyl β-D-galactopyranoside; Sigma, Germany) was used, reactions were carried out at pH 6.6, using 0.1 M Tris-HCl buffer, and Ca^{2+} ions concentration range 5–50 mM. The enzymatic reactions were measured spectrophotometrically at 410 nm using Tecan 96-well plate reader (Tecan Group, Switzerland).The results imply, that Ca^{2+} ions alone do not have effect on lactose hydrolysis, moreover, in some they even stimulate it (NOLA Fit5500). The study results will help to precisely adjust the amount of commercially available enzymes to dairy substrates with high cations content.

Keywords: commercial β-galactosidase, lactose hydrolysis, calcium ions, enzyme kinetics.

Introduction

β-galactosidase (β-D-galactoside, galactohydrolases, EC 3.2.1.23) catalyses the cleavage of terminal galactosyl groups from the non-reducing ends of galactose containing carbohydrates. β-galactosidase is commercially relevant enzyme that is prevalently used for lactose hydrolysis in dairy industry (Carević et al., 2015).

Commercial β-galactosidase preparates are produced from bacterial and eukaryotic hosts: bacteria, yeasts, moulds, (*Bacillus* spp., *Kluyveromyces* spp., *Aspergillus* spp.) (Dagbagli, Goksungur, 2008; Panesar et al., 2006). Enzyme characteristics depends on the source of amino-acids composition, active site and presence or absence of allosteric regulatory sites, pH- and thermal- optimum and stability (Mlichová, Rosenberg, 2006).

Various substances can alter enzyme catalytic activity activating or inhibiting it; metal ions and their complexes are play important role in enzyme structure stabilization and / or activation or inhibition of the reaction. If metal ions are required by enzyme to maintain its stable, native state, it is called as metalloenzyme whereas if metal ions require only during catalytic activity enzymes are called "metal activated enzymes" (Zohra et al., 2016).

Calcium ions (Ca^{2+}) are known as inhibitor for many β-galactosidases, but for some β-galactosidase act also as activator when added in concentrations of 1–10 mM, which also conform to concentrations of free calcium in milk or whey. This property can be advantageous for applications in lactose hydrolysis processes directly in milk or when using lactose-rich substrates based on whey with high level of Ca^{2+} in solution. (Juajun et al., 2011).

The aim of this paper is to determine the impact of Ca^{2+} ions on commercially available β-galactosidase.

Materials and Methods

Chemicals and enzymes

Three commercial preparates of β-galactosidase were used in the study: NOLA Fit5500 and Ha-Lactase 5200 (Chr.HANSEN, Denmark) and Lactozym® Pure 6500 L (Novozymes, Denmark), NOLA Fit5500 is derived from *Bacillus licheniformis*, other two enzymes are of *Kluyveromyces lactis* origin. All enzymes were stored at 4 °C and remained fully active throughout the study All reagents used: $MgCl_2$, $CaCl_2$, p-nitrophenol, 2-nitrophenyl-galactoside (o-NPG) were purchased from Sigma (Germany).

Research was carried out to determinate Ca^{2+} influence at different concentrations (5–50 mM) on β-galactosidase activity of three different, commercially available, enzyme preparates.

Enzymatic assay

β-galactosidase activity was determined using chromogenic substrate o-NPG by monitoring the increasing of absorbance at 410 nm.

Enzyme. Each commercial β-galactosidase were diluted with distillate water for optimal concentration: NOLA Fit5500 with average activity 5500 $BLUg^{-1}$ (1:16); Ha-Lactase 5200, with average activity 5200 NLU g^{-1} (1:2); and Lactozym® Pure6500 L with average activity 6500 LAU g^{-1} (1:4). Activity units of commercial β-galactosidases are defined differently by each producer.

Substrate. All reactions were performed in 0.1 M Tris-HCl buffer, pH 6.6, containing 1 mM $MgCl_2$ and o-NPG concentration in the range from 1.25 to 7.25 mM. To asses Ca^{+2} effect on enzyme activity we used $CaCl_2$ in concentrations 5–50 mM. The measured activities were compared with the activity of the enzyme without added Ca^{+2} under the same conditions.

Enzymatic reactions were measured using 96-well plate in multimode plate reader Infinite 200 M Pro (Tecan Group, Switzerland). Total reaction volume was 200 µL per well, all reactions were started by adding 10 µL of enzyme.

Calibration

o-nitrophenol is hydrolysis product of oNPG. To quantify its production in multimode reader was set up calibration curve by using pure o-nitrophenol solution in the range 0–3.15 mM.

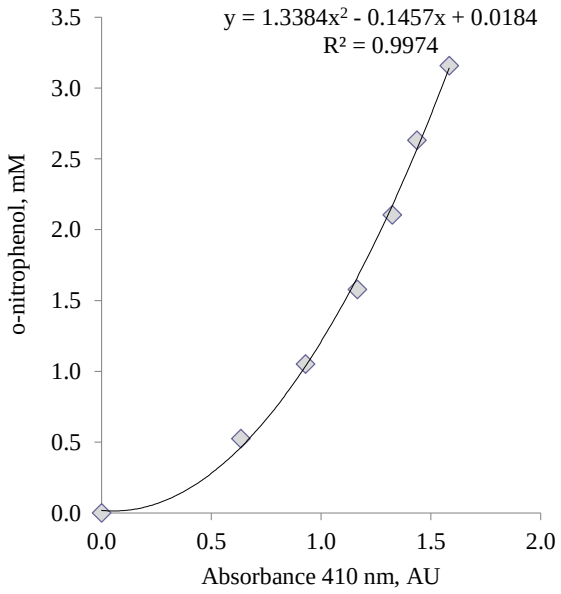

Figure 1. Calibration curve of o-nitrophenol

We found 2nd order polynome approximation to fit our data. By the calibration curve of o-nitrophenol (see Figure 1), were calculated reaction kinetics.

Data analysis

Data were treated by Microsoft Office 2016 Excel.

Results and Discussion

Ca^{+2} is the major cation in bovine milk with a total concentration up to 25 mM (Tanaka et al., 2011). For cheese production, Ca^{+2} is added in concentrations 0.2–0.4 M (Abdalla, Ahmed, 2010; Landfeld et al., 2002; Mehaia, 2006). In whey, cheese by-product, Ca^{+2} concentration can reach 2.0–6.0 mM (Hill et al., 1985; Theoleyre, Gula, 2004; Wong et al. 1978). Due to Ca^{+2} impact potential and its wide range of concentrations in milk and by-products, it is of practical importance to evaluate Ca^{+2} impact on β-galactosidases activity at concentrations up to 50 mM.

Enzyme kinetic constants

To assess Ca^{+2} impact on lactose hydrolysis we studied changes of enzymatic kinetic parameters V_{max} (velocity) and K_M (Michaelis–Menten constant). We used classical Michaelis-Menten type kinetics to describe enzyme activity. For K_M and V_{max} measurement, we used reciprocal graphical representation of enzymatic kinetics (Lineweaver-Burk plots) (Güleç et al., 2010). K_M is equal to the substrate concentration at which the reaction rate is half its maximal value (Atrooz et al., 2016).

To compare the results, all enzyme V_{max} and K_M values were expressed as fold change calculated against respective enzyme kinetic values without Ca^{+2} addition. Results are presented in Table 1.

Table 1

Ca^{2+} effect on commercial β-galactosidase V_{max} and K_m

Ca^{+2}, mM	Lactozym® Pure 6500 L		NOLA Fit5500		Ha-Lactase 5200	
	V_{max}, %	K_M, %	V_{max}, %	K_M, %	V_{max}, %	K_M, %
0	1.000	1.000	1.000	1.000	1.000	1.000
5	1.022	1.421	0.847	0.889	0.011	95.071
10	0.777	*ND	0.836	0.896	0.011	88.906
15	0.834	*ND	*ND	0.782	0.011	91.792
30	0.776	*ND	0.916	0.926	0.011	89.913
50	0.791	*ND	0.859	0.909	0.011	99.612

*ND – not determined

V_{max} and K_M values are presented as fold change when normalised to reaction without Ca^{2+}.

As seen in Table 1, the results showed that commercial β-galactosidases preparates NOLA Fit5500 and Lactozym® Pure 6500 L were rather insensitive to increasing Ca^{2+} concentration, whereas Ha-Lactase 5200 exhibit strong inhibition. Analysing NOLA Fit5500 enzyme activity by changing Ca^{2+} concentration, results showed that oNPG hydrolysis stayed almost the same as it was for the reaction without Ca^{2+} addition. Enzyme kinetic parameters – K_M and V_{max} was the same up to 30 mM of Ca^{2+}. Lactozym® Pure 6500 L showed the highest results at Ca^{2+} concentration 5mM where K_M (1.421%) and V_{max} (1.022%). While at Ca^{2+} concentration up to 10 mM enzyme V_{max} decreased to 0.777% and till 50 mM stayed in similar condition.

Several researches have been made to analyse various monovalent and divalent cations impact on β-galactosidase activity. In the case of β-galactosidase from *Bacillus licheniformis*, results show that Ca^{2+} at concentrations of 1–10 mM together with Na^{+} (1–10 mM) can activate β-galactosidase (Juajun et al., 2011).

Banerjee and co-authors (1982) examined the effect of different metal ion concentrations (Mg^{2+}, Ca^{2+}) on native and immobilized cells of β-galactosidase producing by *Saccharomyces anamensis*, the highest activity was at 2.35 mM concentration. Furthermore it was established that at higher Ca^{2+} concentration the enzyme activity remains unchanged (Banerjee et al., 1982).

We have summarised information from some literature sources on cationic effects on β-galactosidases from various organisms (Table 2). As seen in Table 2, Ca^{2+} can be as β-galactosidase activator or inhibitor.

A wide range of metal ions are known to influence the activity of β-galactosidase (Pandey et al., 2017). The bacterial cell wall contains many types of cations including Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+} (Sahalan et al., 2013).

Table 2

Cation effects on β-galactosidases

Source of enzyme	Activator	Inhibitor	References
Aeromonas caviae	Ca^{2+}; Mg^{2+}	–	(Karunakaran & Devi, 1994)
Kluyveromyces lactis	K^{+}; Mg^{2+}	Ca^{2+}; Na^{+}	(Otieno, 2010)
Lactobacillus reuteri	K^{+}; Na^{+}; Mn^{2+}	Fe^{2+}; Ca^{2+}; Cu^{2+}	(Nguyen et al., 2006)
Bacillus licheniformis	Ca^{2+}; Mn^{2+}; Mg^{2+}	Cu^{2+};Zn^{2+}; Fe^{2+}	(Akcan, 2011)
Kluyveromyces fragilis	Mn^{2+}, Mg^{2+}, K^{+}	Ca^{2+}	(Mlichová & Rosenberg, 2006)
Amygdalus communis	Ca^{2+}; Mn^{2+}	K^{+}; Na^{+}	(Pal et al., 2013)
Saccharomyces anamensis	Ca^{2+}; Mn^{2+}	–	(Banerjee et al., 1982)

Bacterial β-galactosidase can be activated by Ca^{2+}, while yeast or mould β-galactosidase for most of the cases – inhibited (see Table 2) (Mlichová, Rosenberg, 2006). In some cases, Ca^{+2} and Mg^{+2} is absolute necessity for β-galactosidase activity of *Aeromonas cauiae* (Karunakaran, Devi, 1994). Instead Kumar and co-authors (2015) stated that metal ion Ca^{2+} and Na^{+} in concentration of 10–30 mM did not affected the *Serratia quinivorans* β-galactosidase activity (Kumar et al., 2015).

It should be noted that nowadays the commercial β-galactosidase is mainly produced by *Kluyveromyces lactis* (You et al., 2017) such as Lactozyme 2600 L, GODO-YNL2, Ha-Lactase 5200, Lactozym® Pure 6500 L and Maxilact® LX5000 which is used in industrial field.

Conclusions

The results imply, that Ca^{2+} alone does not have effect on lactose hydrolysis by NOLA Fit5500 and Lactozym® Pure 6500 L β galactosidases, enzyme kinetic parameters – K_m and V_{max} at different Ca^{2+} concentrations stayed almost the same as it is for the reaction without Ca^{2+} addition. Ha-Lactase 5200 β-galactosidase is strongly inhibited by Ca^{2+}, this preparate would not be recommended to use for lactose hydrolysis in media with a high concentration of Ca^{2+}. Future research should be done to find out if Ca^{2+} together with other cations present in whey has additive effects on β-galactosidase activity.

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