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Is divalent magnesium cation the best cofactor for bacterial β-galactosidase?

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β-Galactosidase is a metal-activated enzyme, which breaks down the glucosidic bond of lactose and produces glucose and galactose. Among several commercial applications, preparation of lactose-free milk has gained special attention. The present objective is to demonstrate the activity kinetics of β-galactosidase purified from a non-pathogenic bacterium *Arthrobacter oxydans* SB. The enzyme was purified by DEAE-cellulose and Sephadex G-100 column chromatography. The purity of the protein was checked by high-performance liquid chromatography (HPLC). The purified enzyme of molecular weight ~ 95 kDa exhibited specific activity of 137.7 U mg^{-1} protein with a purification of 11.22-fold and yield 12.42 %. The exact molecular weight (95.7 kDa) of the purified protein was determined by MALDI-TOF. Previously, most of the studies have used Mg^{+2} as a cofactor of β- galactosidase. In this present investigation, we have checked the kinetic behavior of the purified β-galactosidase in presence of several bivalent metals. Lowest K_m with highest substrate (ortho-nitrophenyl-β-galactoside or ONPG) affinity was measured in presence of Ca^{2+} (42.45 μM ONPG). However, our results demonstrated that V_{max} was maximum in presence of Mn^{+2} (55.98 μM ONP produced mg^{-1} protein min^{-1}), followed by Fe^{+2}, Zn^{+2}, Mg^{+2}, Cu^{+2} and Ca^{+2}. A large number of investigations reported Mg^{+2} as potential co factor for β-galacosidase. However, β-galactosidase obtained from *Arthrobacter oxydans* SB has better activity in the presence of Mn^{+2} or Fe^{2+}.

Keywords. Bivalent metals; kinetic behavior; MALDI-TOF; purification; β-galactosidase

1. Introduction

β-Galactosidase (commonly known as lactase) is an important enzyme which hydrolyzes the glycosidic linkage of lactose and produces glucose and galactose. β-Galactosidase is widely distributed in different living organism, starting from lower prokaryotes like bacteria, fungi to higher eukaryotes (Husain 2010; Juers *et al.* 2012). Nowadays this enzyme is frequently used in different food industries for flavor enrichment and sweetness improvement (Grosova *et al.* 2008; Juers *et al.* 2012). Furthermore, lactose intolerance problem is handled to a great extent using lactase, which cleaves beta-l, 4 linkages of lactose and releases simple sugars (Husain 2010). Another important modern utilization of the enzyme is as biosensor to detect the lactose in milk (Marrakchi *et al.* 2008). Until now, *Bacillus subtilis*, *Bifidobacterium longum*, *Streptococcus thermophilus, Lactobaccillus*, *Kluyveromyces lactis* and *Aspergillus niger* were considered to be the commercial producer of β-galactosidase in different industries (Saishin *et al.* 2010; Oliveira *et al.* 2011; El-Kader *et al.* 2012; Prasad *et al.* 2013). However, production of lactose-free milk with purified enzyme increases the production cost up to 80% as compared to regular milk (Bury and Jelen 2000). Therefore, continuous screening and isolation of β-galactosidase-producing potential candidates and enhancement of the enzyme activity by biochemical/protein engineering is a never ending process.

In general, β-galactosidase activity is measured in Z buffer containing Mg^{+2} salt as the activator. The magnesium is reported to be the conventional cofactor of the enzyme β-galactosidase (Richard *et al.* 1995; Juers *et al.* 2012). So, the question is, is Mg^{+2} really the best cofactor for β-galactosidase? The present study elucidates the activity of several bivalent metal cofactors supplementation, compared to the conventional use of magnesium as a cofactor. Here we have demonstrated the kinetic behavior of a novel β-galactosidase enzyme purified from a non-pathogenic bacteria *Arthrobacter oxydans* SB in the presence of several metal cofactors.

2. Materials and methods

2.1 *Bacterial strain and extraction of intracellular β-galactosidase*

The bacterial strain used for the source of β-galactosidase was *Arthrobacter oxydans* SB (Banerjee *et al*. 2016). The extraction of intracellular β-galactosidase was done following the method of Banerjee *et al*. (2016). In brief, *A*. *oxydans* SB was cultured in 500 ml of lactose broth for 24 h at 30°C. The bacterial cells were harvested by centrifugation (8000*g*, 10 min) at 4°C, resuspended in 30 ml Z buffer mixed with 100 µl of tween-20 and incubate for 12 h at room temperature. Supernatant containing the crude enzyme was collected by centrifugation (10,000 × g, 10 mins) at 4°C and store at −20°C for further experiment.

2.2 *Purification of β-galactosidase*

β-Galactosidase was first precipitated by adding solid ammonium sulphate (20%, 40%, 60% and 80% saturation) at 4°C and kept overnight. Precipitated protein was collected by centrifugation (15,000*g*, 15 min) at 4°C, suspended in 50 nM phosphate buffer (pH 7.0) and dialyzed over night against the same buffer to remove the excess ammonium sulphate. β-Galactosidase was then purified in anion exchange chromatography (DEAE cellulose), followed by gel filtration chromatography (Sephadex G-100). Active fraction was pooled and store for further study. The purity of the purified protein was finally confirmed by HPLC. Homogeneity of the purified enzyme was determined by SDS-PAGE using molecular weight marker (Fermentas Cat. No. SM-0671) as a standard.

2.3 *MALDI-TOF analysis of the purified enzyme*

The sample preparation for MALDI-TOF analysis was done following the method of Shevchenko *et al*. (2006). In brief, sample was digested using trypsin solution (12.5 ng μl^{-1} - trypsin in 50 mM NH_4HCO_3) and subjected to MALDI-TOF (MALDI-TOF mass spectrometer equipped with Applied Biosystems 4700 Proteomics Discovery System, Darmstadt, Germany) analysis.

2.4 *Determination of activity kinetics of β-galactosidase*

β-Galactosidase assay was performed using ONPG (o-nitrophenyl-β-D- galactopyranoside) as substrate in 50 mM Z buffer (pH 7.0). 30 µl of enzyme was mixed in 160 µl of substrate and 700 µl of Z buffer was added. The reaction mixture was incubated for 30 min at 37°C and reaction was stopped by adding 400 µl of 1 M Na_2CO_3. Amount of ortho-nitro phenol (ONP) released from ONPG was determined by measuring OD at 420 nm. The activity was calculated using the molar extinction coefficient of ONP (2.13×10^4 M^{-1} cm^{-1}). The specific activity was expressed in term of micromolar ONP produced mg^{-1} protein min^{-1}. The β-galactosidase assay was also performed under varied substrate concentration of 30, 60, 120, 240, 480 and 960 µM of ONPG to evaluate the influence of different metals cofactor (Ca^{+2}, Fe^{+2}, Mn^{+2}, Mg^{+2}) at a concentration of 8 mM, except Cu^{+2}, Zn^{+2} (6 mM) on the enzyme activity (table 1). The K_m and V_{max} were calculated from non-liner analyses of Michaelis–Menten kinetics. Protein was estimated according to the method described by Lowry *et al*. (1951) using BSA as standard.

3. Results and discussion

3.1 *Purification and characterization of β-galactosidase*

The highest activity of the β-galactosidase was achieved at 60% ammonium sulphate saturation level (40.98 U mg^{-1}

Table 1. Enzyme activity at different concentration of metal ions

	β-Galactosidase activity (U mg^{-1} protein) (±SD)					
Metal conc. (mM)	Fe^{+2}	Zn^{+2}	Mn^{+2}	Mg^{+2}	Cu^{+2}	Ca^{+2}
4	23.59 (±1.1)	18.92 (± 0.7)	22.45 (±1.2)	19.56 (±1.2)	19.06 (±0.9)	11.32 (±0.8)
6	24.72 (± 1.3)	20.3 (±1.1)	24.61 (±1.0)	20.71 (±0.9)	21. 87(±1.1)	12.81 (± 0.7)
8	25.16 (± 1.1)	19.11 (±0.9)	24.82 (±0.9)	21.29 (±0.8)	20.51 (±1.2)	13.24 (±1.0)
10	12.29 (± 0.7)	7.45 (±0.4)	11.07 (±0.6)	13.83 (±1.1)	9.44 (±0.5)	5.01 (±0.4)

Data are represented as mean of triplicate value ± standard deviation (SD)

U = µM *o*-nitro phenol (ONP) production min^{-1}

Table 2. Details purification steps of β-galactosidase (activity assay was checked traditionally in presence of Mg^{+2})

Purification step	Total protein content (mg)	Total enzyme activity (U)	Specific activity (U mg^{-1} protein)	Purification (Fold)	Yield (%)
Crude	119.21	1462.70	12.27	1.0	100
$(NH_4)_2SO_4$	31.25	1280.68	40.98	3.33	87.50
Dialysis	18.34	974.03	53.10	4.18	66.59
DEAE-Cellulose	4.16	481.27	115.68	9.42	32.9
Sephadex G-100	1.32	181.77	137.70	11.22	12.42

U = μM *o*-nitro phenol (ONP) production min^{-1}

protein). The stepwise purification of the β-galactosidase is given in table 2. The purified β galactosidase exhibited a specific activity of 137.7 U mg^{-1} protein with a purification fold 11.22 and yield 12.42 %. As already mentioned, β-galactosidase is an important enzyme in the food industry and therefore, several investigations have been conducted to isolate and characterize β-galactosidase from different sources such as bacteria (Saishin *et al*. 2010; Princely *et al*. 2013), fungi (Hu *et al*. 2010), plant (Panesar *et al*. 2010), animal (Asraf and Gunasekaran 2010), and yeast (Viana *et al*. 2006; Husain 2010). However, the characteristic and activity of the β-galactosidase obtained from different sources varied greatly (Husain 2010). In the present study, the apparent homogeneity and molecular weight of the purified β-galactosidase was ∼ 95 kDa (figure 1). The purity of the final product was confirmed by HPLC. The MALDI-TOF analysis demonstrated that the purified protein was 95.7 kDa with no subunit. Saishin *et al*. (2010) purified β-galactosidase from *Bifidobacterium longum* and reported the molecular weight as 77 kDa and 110 kDa in SDS PAGE and native PAGE, respectively. Furthermore, Bhalla *et al*. (2015) purified a β-galactosidase (60 kDa) from *Lactobacillus brevis* PLA28 using two step purification methods; ammonium sulphate precipitation method and hydrophobic interaction chromatography, respectively. While, Badarinath and Halami (2011) have reported a novel β-galactosidase (90 kDa) from *Enterococcus faecium* MTCC5153, which contains two homodimeric subunits of 43 kDa. To the author's best knowledge, it the first report of 95.7 kDa β-galactosidase from *Arthrobacter oxydans* SB.

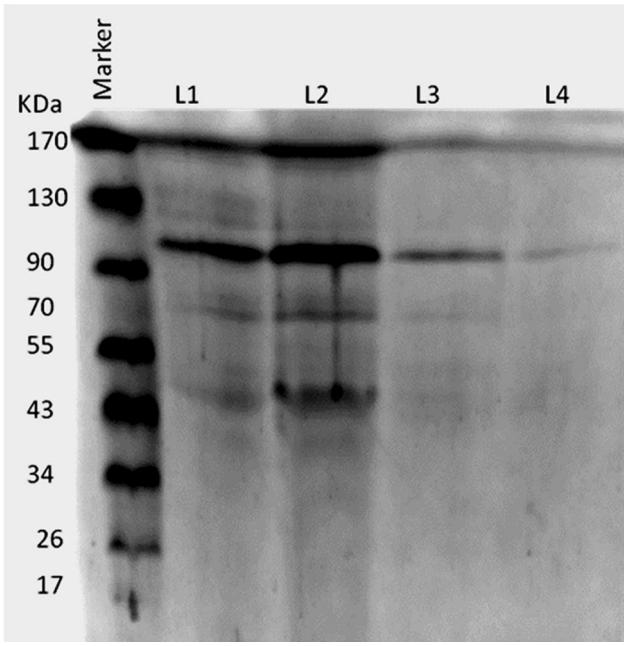

Figure 1. Represents the homogenicity of purified β-galactosidase in SDS-PAGE. Lane 1, 2, 3 and 4 represent the purification at different steps; ammonium sulphate precipitation, DEAE cellulose, Sephadex G100 and final fraction, respectively. The molecular weight of purified β-galactosidase is ∼ 95 kDa.

3.2 *Kinetic behavior of β-galactosidase*

The nonliner analyses of Michaelis–Menten kinetics depicts that maximum rate of reaction (V_{max}) of β-galactosidase enzyme was achieved in the presence of Mn^{+2} (55.98 μM ONP produced mg^{-1} protein min^{-1}) (figure 2A), followed by Fe^{+2} (53.0 μM ONP produced mg^{-1} protein min^{-1}) (figure 2B) as compared to the effects of other metallic ions (figure 2C–F). Concomitant to this observation, we found that the K_m value of the enzyme due to Mn^{+2} was 969.7 μM. Similarly supplementation of other metallic cations as predicted cofactors including Zn^{+2}, Mg^{+2}, Cu^{+2} and Ca^{+2} demonstrates gradual decreasing activity expressed in V_{max} (figure 2). However, the K_m value of those metals supplemented reaction mixture was found to be 577.0, 423.9, 253.9, and 42.45 μM ONPG, respectively (table 3). Reduction of K_m in presence of Ca^{2+} fairly exhibits increase

Table 3. The details of reaction velocity and substrate affinity of the selected metals

Metal ions	Reaction velocity (V_{max}) U1	Substrate affinity (K_m) U2
Fe^{+2}	53.00	926.1
Zn^{+2}	36.98	577.0
Mg^{+2}	30.77	423.9
Mn^{+2}	55.98	969.7
Ca^{+2}	13.03	42.45
Cu^{+2}	20.10	253.9

U1 = μM ONP Produced mg^{-1} protein min^{-1}

U2= μM ONPG

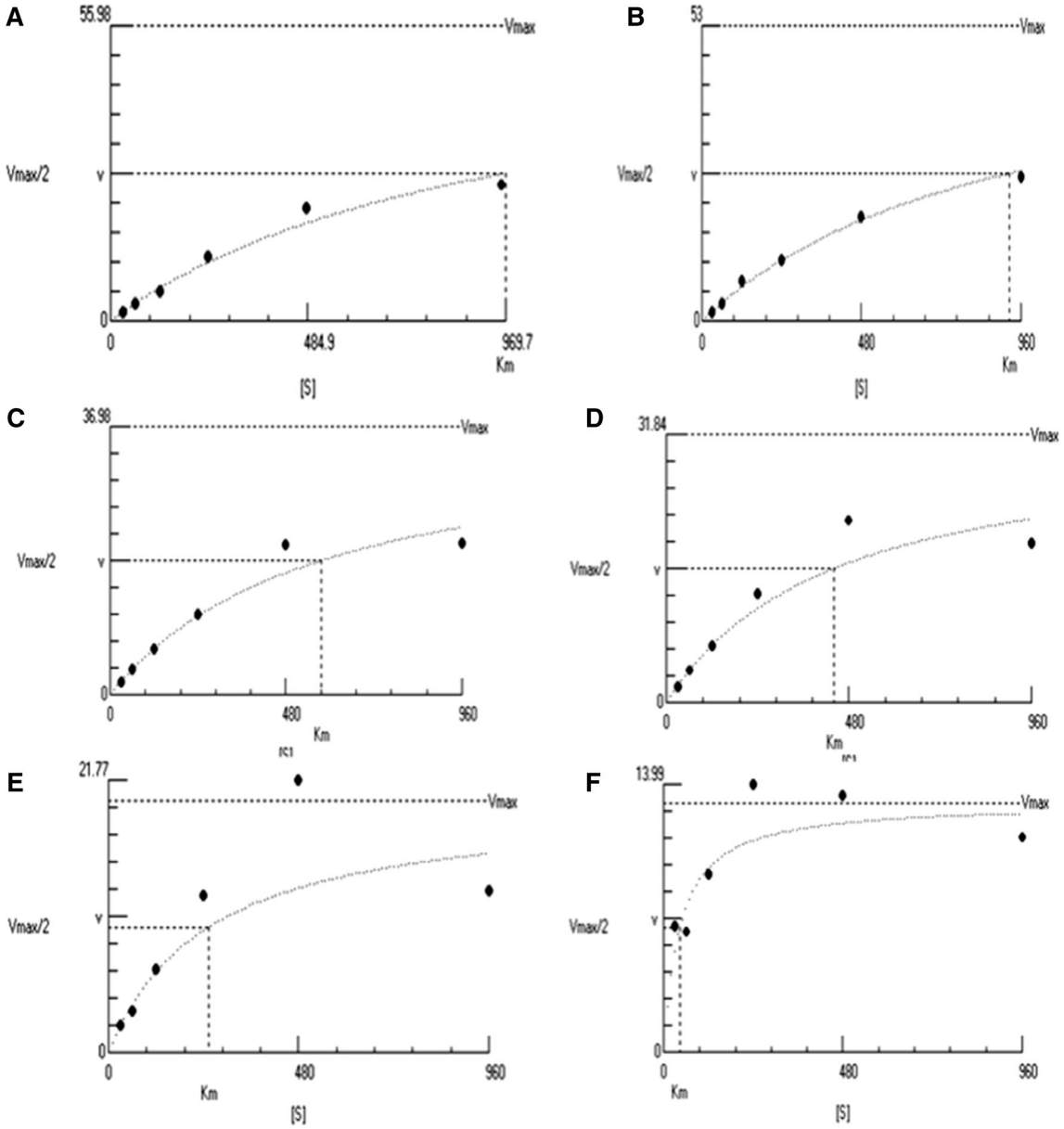

Figure 2. Nonliner analyses of Michaelis–Menten kinetics of β galactosidase activity purified from *Arthrobacter oxydans* SB in presence of different bivalent metal. (**A**) Mn^{+2}, (**B**) Fe^{+2}, (**C**) Zn^{+2}, (**D**) Mg^{+2}, (**E**) Cu^{+2} and (**F**) Ca^{+2}. The plot demonstrates the maximum rate of reaction (V_{max}) was achieved in the presence of Mn^{+2} as compared to the other metallic ions, where K_m value of the enzyme due to this bivalent cation was found to be 969.7 μM. Similarly, supplementation of other metallic cations as predicted cofactors including Zn^{+2}, Mg^{+2}, Cu^{+2} and Ca^{+2} demonstrates gradual decreasing activity expressed in V_{max}.

in substrate affinity, however concomitant reduction in V_{max} apparently reflects an overall depiction of uncompetitive mode of alteration (inhibition/activation) as compared to other ions. Therefore, it is stated that considering maximum velocity, Mn^{+2} and/or Fe^{+2} can be used as better cofactor of the enzyme in present case. Until now, majority of reports have been published on the kinetic nature of β-galacosidase at different temperature and pH (Princely et al. 2013; Bhalla *et al*. 2015). However, reports on kinetic behavior β-galacosidase in presence of divalent cations are scanty. In this study, we demonstrate the K_m and V_{max} of the purified β-galacosidase in presence of different cations. A large number of investigations reported Mg^{+2} as potential co factor for β-galacosidase (Loveland et al. 1994; Griffith and Wolf 2002; Dhaked *et al*. 2005; Manera *et al*. 2008; Princely *et al.* 2013). However, β-galacosidase isolated and purified from *A*. *oxydans* SB exhibited highest activity (in terms of V_{max}) in presence of Mn^{+2} as well as Fe^{+2} ions.

4. Conclusion

With the results of the present findings, a combination of metal ions especially Mn^{+2} or Fe^{+2} and Ca^{+2} can be tested in future to obtain the optimum but not maximum activity in practice. Furthermore, there is ample scope for cofactor optimization with combinations of bivalent metals, which will enhance the β-galacosidase activity and reduce the production cost of lactose-free milk the industry.

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