Effects of Major Ingredients in Cattle Milk on Enzyme Kinetics of Recombinant β-galactosidase (BGalP) Expressed in Pichia pastoris

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Abstract

Background and objective: β-galactosidase enzymes hydrolyze lactose into glucose and galactose for production of lactose free dairy products. However, different ions and fat content in milk may act as the inhibitor or activator for β-galactosidase enzymes. A cold-active β-galactosidase enzyme (BGalP), originally from Planococcus sp. LA4, was previously expressed in Pichia pastoris to perform lactose hydrolysis in the refrigerated milk. In this study, the effects of milks major ingredients were evaluated on the enzymatic kinetics to confirm its capacity for hydrolyzing milk lactose.

Material and methods: The activity was determined in different concentrations of NaCl, KCl, MgCl2, and CaCl2 as well as in the milk with low, medium or high-fat content. In these experiments ortho-Nitrophenyl β-galactoside was used as the substrate. Additionally, glucose was measured as the product after incubation of milk with BGalP enzyme for 24 h at room temperature.

Results and conclusion: This study demonstrated that ions and fat content did not adversely affect the enzyme activity in the concentration corresponding to the milk contents. Ca (27.5-32.5 mM), Cl (25.3-30.9 mM), Na (15.2-39.1 mM) and Mg (3.75-5.83 mM) had no inhibitory effects, but KCl decreased the enzyme activity. Since Cl existed in MgCl2 and CaCl2 exerted no inhibitory effects, it can be concluded that inhibitory effects of KCl resulted from potassium rather than chloride. The results indicate that BGalP enzyme was not inhibited by milks major ingredients and has the potential to be used for the production of lactose-free dairy products.

Conflict of interest: The authors declare no conflict of interest.

1. Introduction

Milk is a rich source of macro and micronutrients; however, lactose intolerance, affecting nearly 70% of the population, discourages many adults from milk consumption [1-5]. The common solution is production of lactose-free milk and other dairy products by using β-galactosidase for hydrolysis of lactose into glucose and galactose [6]. This process increases sweetness of the product and reduces sugar consumption in the dairy products [5]. β-galactosidase enzymes have been also used for synthesis of galacto-oligosaccharides as probiotics [7,8]. These enzymes are naturally expressed in different plants, animal cells and more commonly in microorganisms such as bacteria, yeast, and fungi [5]. The most important industrial sources of β-galactosidase are Kluyveromyces (K.) fragilis, K. lactis, Aspergillus (A.) niger and Escherichia (E.) coli [9,10]. However, due to low levels of expression, its extraction from these natural sources is not economical. Alternatively, production of an enzyme as a recombinant protein can reduce the production costs [11]. Cold-active β-galactosidase (BGalP) enzymes have been considered for lactose hydrolysis in refrigerated milk, which was reported to be highly active for lactose hydrolysis at 5 and 20°C [12,13]. Such psychrophilic enzyme, originally from Planococcus sp. LA4, was previously expressed as a recombinant protein in Pichia (P.) pastoris in our laboratory [14]. However, its kinetic properties were yet to be investigated. The enzymes isolated from various sources have different properties including molecular weight, metal ion requirement, and the pH and temperature for their optimum
activity [15]. Depending on the source of the enzyme, metal ions, glucose and galactose may differently affect enzymatic activity of the β-galactosidase. For example, activity of the enzyme isolated from K. lactis was increased in presence of metal ions such as Manganese and was inhibited in the presence of imidazole above 50 mM, as well as galactose and Ca [16,17]. Glucose has been reported as an activator for the β-galactosidase enzyme [18]. While the β-galactosidase extracted from Bacillus (B.) licheniformis DSM 13 and E. coli have been inhibited strongly in the presence of glucose and galactose, their activity was increased with metal ions K and Na (1-100 mM), Mg, Mn and Ca (1 mM) [19].

The activity of the enzyme extracted from Fusarium oxysporum is inhibited at the presences of divalent cations as Zn, Mg and Ca [20]. The stability and activity of the β-galactosidase from Lactobacillus pentosus was increased at presences of Mg [21]. The enzyme isolated from Enterobacter cloacae is activated by Mg, but inhibited by Pb and Zn [22]. Since lactose hydrolysis results in galactose production and Ca is also one of the main naturally occurring ingredients in milk, these enzymes may not be suitable for the production of lactose-free milk [23]. In this study, the effects of different metal ions and fat content were investigated on the kinetics of the BGalP enzyme to evaluate its capacity for production of lactose-free dairy products.

2. Materials and methods

BGalP expression

The P. pastoris, previously transformed to express BGalP, was cultured in buffered complex medium with glycerol (BMGY) containing yeast extract (10 g 1⁻¹) (Himedia, India), peptone (20 g 1⁻¹) (Sigma-Aldrich, USA), yeast nitrogen base (13.4 g 1⁻¹) (Sigma-Aldrich, USA), glycerol (10% v⁻¹) (Merck, Germany), biotin 0.4 mg 1⁻¹ (Alborzdaru, Iran) in 100 mM potassium phosphate buffer (pH 6.0) (Merck, Germany) [14]. When the OD₆₀₀ reached 1.5, the medium was replaced by buffered complex medium containing methanol (BMMY). This medium contained the similar ingredients to BMGY except for the glycerol which was replaced by 2% v⁻¹ methanol (Merck, Germany) to stimulate expression of the BGalP protein. The expression was continued for 5 days and the supernatant concentrated 4-times using a 10 kDa MVCO (Merck, Germany).

Enzyme activity assay using ONPG as the substrate

The enzyme activity in the culture supernatant was measured in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol (Merck, Germany), pH 7.0) and ortho-Nitrophenyl β-galactoside (ONPG) (Sigma-Aldrich, USA) as the substrate as previously described (18). The absorbance was measured using a Victor X5 plate reader (Perkins Elmer, Waltham, MA). As recommended in the manual for Pichia Expression Kit (Invitrogen, catalog no. K1710-01), β-galactosidase activity (IU m⁻¹ of the culture supernatant) was calculated by multiplying OD₄₂₀ change in each minute by 380 (a constant value related to the ONPG extinction coefficient). The only modification in the calculation method was to normalize the enzyme activity to the culture supernatant volume (ml⁻¹) rather than its protein content (mg⁻¹).

Evaluating the effect of metal ions on BGalP activity

Stock solutions of KCl (100 mM), NaCl (88 mM), MgCl₂ (21 mM) and CaCl₂ (130 mM) (all from Merck, Germany) were prepared in Z buffer. These stocks contained the concentration of ions nearly 4 times more than milk and were diluted serially in Z buffer for measuring BGalP enzyme activity.

Comparing BGalP activity in Z buffer and deionized water

In a 96-well plate, serial dilutions of lactose (Merck, Germany) were prepared in deionized water and Z buffer. Then 20 µl of culture supernatant was added to each well. After 24 h incubation at room temperature, the concentration of glucose was evaluated according to the product using Pars Azmun kit (Pars Azmun kit, Iran). The enzyme activity was calculated as micromole(s) of glucose produced per minute.

Investigating the inhibitory effect of lactose on BGalP activity

The BGalP enzyme activity was determined in presence of 5% lactose and different concentrations of ONPG.

Effect of milk fat concentrations on recombinant BGalP activity

The enzyme (20 µl of the culture supernatant) was added to 100 µl of commercially packed milk with different concentrations of fat (1.5, 2.5, 3%). After 24 h incubation at room temperature, the concentration of glucose was measured, the enzyme activity (IU min⁻¹) was calculated as micromole(s) of glucose produced per minute.

Statistical analysis

The final results were analyzed by GraphPad Prism software 7 for Windows (GraphPad Software, San Diego, CA). The data was analyzed using nonlinear regression fit and Michaelis-Menten enzyme kinetics model \( V=V_{max}[S] \ (K_{m}+[S])^{-1} \) to calculate \( K_m \) and \( V_{max} \) values. The mean values calculated from three independent experiments were compared using ANOVA in SPSS 16 software package (SPSS Inc, IL) and considered significantly different if the p-value were ≤0.05. Data presented is expressed as Mean±SD.
3. Results and discussion

BGalP was produced in *P. pastoris* in an attempt to preserve nutrients and taste, increasing process flexibility and reducing the cost of manufacturing lactose-free milk through performing lactose hydrolysis at refrigerated temperatures [14]. This is a preliminary investigation aiming to determine whether BGalP is an appropriate enzyme for the production of lactose-free milk.

The effects of metal ions on BGalP activity

The enzyme activity at buffer Z [recommended as optimal condition in the manual for Pichia Expression Kit (Invitrogen, catalog no. K1710-01)] were 1482 IU ml⁻¹. The BGalP activity was not adversely affected by MgCl₂ at the concentrations up to 21 mM. In contrast, adding KCl led to about 2 fold decrease of enzyme activity (Fig. 1). These results indicate that Mg²⁺ and Cl⁻ ions, unlike K⁺, did not exert any inhibitory effects of BGalP activity at the concentrations up to 4 times higher than those naturally occurring in cow milk.

![Figure 1](image1)

**Figure 1.** Evaluating the effects of different concentrations of KCl and MgCl₂ on enzyme activity. The enzyme activity was determined in Z buffer containing KCl (3.12-100 mM) and MgCl₂ (0.325-20.8mM). Ortho-Nitrophenyl β-galactoside was used as the substrate. BGalP activity was calculated by multiplying OD₅₇₀ change in each minute by 380 (a constant value related to the ortho-Nitrophenyl β-galactoside extinction coefficient) and finally its normalization to the culture supernatant volume added to the reaction. The experiments were repeated three times and their mean was reported as IU ml⁻¹ of the culture supernatant.

There are some inconsistent reports on effects of magnesium on enzyme activity of the β-galactosidase extracted from *Arthrobacter* sp. Mg²⁺ acted as an inhibitor for the enzyme extracted from *Arthrobacter* sp.32c, but as an activator for the enzyme derived from *Arthrobacter* sp. B7 [12,24].

The β-galactosidase from *Planococcus* sp. L4 was expressed in *E. coli* and its *Kₘ* for ONPG was 2.9 mM; which is consistent with 3.4 observed in this study [17]. When the β-galactosidase from halotolerant *Planococcus* sp. L4 was expressed in *E. coli*, it was resistant to high concentrations of K⁺ and Na⁺ [17]. However, at high concentrations, Na⁺ and Ca²⁺ ions act as inhibitors for BGalP (Fig. 2). The enzyme activity was inversely correlated with NaCl concentrations. CaCl₂ also inhibited the activity of BGalP at the concentrations above 32 mM. The result of this experiment also supported the previous conclusion that the Cl⁻ ion is not a BGalP activity modulator. Previous studies also showed that Ca²⁺ ion inhibited the enzymes derived from *Arthrobacter* sp. B7 and sp. 32c [24,26]. It is consistent with the results of this study, in which high concentrations of Na⁺ and Ca²⁺ ions inhibited the BGalP activity (Fig. 2).

![Figure 2](image2)

**Figure 2.** Evaluating the effect of different concentration of NaCl and CaCl₂ on enzyme activity. BGalP enzyme activity was measured in serial dilutions of NaCl (1.3-87 mM) and CaCl₂ (1-130 mM) were prepared in Z buffer. Ortho-Nitrophenyl β-galactoside used as the substrate. BGalP activity was calculated by multiplying OD₅₇₀ change in each minute by 380 (a constant value related to the ortho-Nitrophenyl β-galactoside extinction coefficient) and finally its normalization to the culture supernatant volume added to the reaction. The experiments were repeated three times and their mean was reported as IU ml⁻¹ of the culture supernatant.

BGalP hydrolyzed lactose with the *Vₐₓₜₜ* of 0.001 IU ml⁻¹ and its half-saturation coefficient (*Kₘ*) was 124.4 mM in Z buffer. Similarly, a higher *Vₐₓₜₜ* (0.0028 IU ml⁻¹) and lower *Kₘ* (94.25 mM) observed in deionized water. This result indicated that Z buffer ingredients could adversely affect the activity of BGalP by decreasing the *Vₐₓₜₜ* and increasing the *Kₘ* (Fig. 3). Additional experiments confirmed that the activity of BGalP enzyme in Z buffer was lower compared to deionized water that may be due to the presence of sodium (as Na₂HPO₄ and NaH₂PO₄) in Z buffer.
Substrate specificity of BGalP

BGalP demonstrated relatively high affinity toward ONPG (indicated by its low $K_{m}$) when compared to the other β-galactosidase enzyme extracted from different sources (Table 1).

Table 1. $K_{m}$ value reported for β-galactosidase enzymes originated from different organisms. The assay was performed using ONPG as the substrate.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>$K_{m}$ (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGalP expressed in <em>Pichia pastoris</em></td>
<td>3.97</td>
<td>Current work</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>65.36</td>
<td>[28]</td>
</tr>
<tr>
<td>Planococcus sp. L4</td>
<td>2.9</td>
<td>[17]</td>
</tr>
<tr>
<td>Arthrobacter sp. B7 gene 15</td>
<td>0.4</td>
<td>[24]</td>
</tr>
<tr>
<td>Arthrobacter psychrolactophilus F2</td>
<td>2.8</td>
<td>[29]</td>
</tr>
<tr>
<td>Paracoccus sp. 32d</td>
<td>1.17</td>
<td>[30]</td>
</tr>
<tr>
<td>Pseudoalteromonas sp. TAE 79b</td>
<td>0.16</td>
<td>[31]</td>
</tr>
<tr>
<td>Flavobacterium sp. 4214</td>
<td>0.65</td>
<td>[32]</td>
</tr>
<tr>
<td>Guehomyces pullulans 17-1</td>
<td>3.3</td>
<td>[33]</td>
</tr>
<tr>
<td>Halomonas sp. S62</td>
<td>2.9</td>
<td>[34]</td>
</tr>
</tbody>
</table>

To reveal the substrate specificity of the enzyme, $K_{m}$ and $V_{max}$ of BGalP were determined using ONPG as the substrate in presence of 5% lactose. Lactose increased the $K_{m}$ for ONPG from 3.97 to 14.56 mM. In lactose-free assay, at ONPG concentration of 0.8 mM, the enzyme activity was 321 IU ml$^{-1}$; which declined to 66 IU ml$^{-1}$ in presence of 5% lactose which could only be restored by 6.25 mM ONPG. This suggests that lactose may compete with ONPG for binding to the BGalP catalytic site (Fig. 5).

These results attested that BGalP enzyme may have higher affinity for binding to lactose than ONPG.

Substrate specificity of BGalP

It was previously reported that MgCl$_2$ (10 mM), CaCl$_2$ (10 mM) and MnCl$_2$ (10 mM) did not have any adverse effects on the activity of the enzyme isolated from *E. coli*. KCl and NaCl activated the enzyme at low concentrations (250 mM) but acted as inhibitors at higher concentrations. The enzyme activity decreased in the presence of 1 mM concentrations of NiCl$_2$, CuSO$_4$, ZnSO$_4$ and CaCl$_2$ [27]. Some of these ions do not occur in significant concentrations in the cattle milk; therefore they were exempted from the current study. BGalP, however, was highly sensitive to the inhibitory effects of the Na and K ions which are different from the enzyme extracted from *E. coli*. Sequence alignment revealed only 10% identity between these two enzymes, which may be the cause of these variations in kinetics properties (Fig. 4).

**Figure 3.** Comparing kinetic parameters of BGalP in Z buffer and deionized water. Enzyme activity was reported as micromole glucose produced per minute of reaction.

**Figure 4.** Amino acid sequences alignment of the enzyme from *Escherichia coli* (BGAL-ECOLI) with BGalP (BGAL-PLASL); It was performed using Geneious software suite. Identical and similar amino acids are indicated by black and gray backgrounds, respectively.
Milk ingredients effects on BGalP Kinetics

**Figure 5.** Substrate specificity of BGalP. BGalP activity was measured in serially dilution concentration of ONPG (0.4-25 MM) as the substrate in the reaction medium with or without 5% lactose.

**Effect of milk fat content on BGalP activity**

As shown in Fig. 6, there was no significant difference in enzyme activity values in the milk containing 0, 1.5, 2.5, or 3% fat (p>0.05). This can be considered as an important advantage for BGalP which is going to be used for lactose hydrolysis in milks containing different levels of fat.

**Figure 6.** Effect of milk fat on enzyme activity. The enzyme activity (IU min⁻¹) was calculated as micromole(s) of glucose produced per minute.

4. Conclusion

It seems that none of the naturally occurring β-galactosidase enzymes have all the ideal properties to be used for production of lactose-free dairy products. The main drawbacks include inappropriate optimum pH and being adversely affected by the milk ingredients. Interestingly, in this study, it was confirmed that at naturally accruing concentrations of milk ingredients, the fat content and metal ions, excluding potassium, did not have any significant inhibitory effects on BGalP. Therefore, BGalP can potentially be used for production of lactose-free dairy products. Its kinetic properties can also be improved by removing the glycosylation or even protein engineering techniques, if required.

5. Acknowledgements

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6. Conflict of interest

The authors declare that they have no conflict of interest with this publication.

References


اثرات اجزای مهم شیر گاو بر کینتیک آنزیم نوترکیب بتا-گالاکتوزیداز (BGalp) تولید شده در 
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چکیده
سابقه و هدف: آنزیم بتا-گالاکتوزیداز موجب آکتیوکسیژن آلکوژن و تولید فرآوردهای شیری بدنی
لایه می‌شود. میزان جریب و بی‌پویانی گوناگون در شیر می‌تواند به عنوان مارک‌گذاری با گالاکتوزیداز بنا- 
کینتیک فعالیت کند. در مطالعه قبلی Zn آنزیم بتا-گالاکتوزیداز فعال در سرمای (BGalP) در پایین‌پوشک (sp. L4) 
با سه‌پایی پستوریس بیان و با حساسیت بین آن‌گونه‌ای آکتفکس شد. در این مطالعه، برای تایید فرضیه این آنزیم در آکتفکس بین هایی (BGalP) با تأثیر فعال آن کینتیک آنزیمی 
در سطح عالی است.

مواد و روش‌ها: فعالیت آنزیم (BGalP) در غلظت های گوناگون از بینهای سه سالم لایه (NaCl) و 
کلسیم کلرید (CaCl2) و مسیر کلرید (KCl) و در شیر حاوی مقادیر کم، متوسط و زیاد (میکرو)
و در شیر حاوی مقادیر کم، متوسط و زیاد (میکرو)
و در شیر حاوی مقادیر کم، متوسط و زیاد (میکرو)
و در شیر حاوی مقادیر کم، متوسط و زیاد (میکرو) 
مورد استفاده قرار گرفت. 
همچنین، داده‌های چهارمتوکانسولار در دمای آتش میزان غلظت گلوك تولید شده به عنوان فرآورده به دست آمده 
ادناره‌گری شد.

یافته‌ها و نتیده‌گری: در مطالعه نشان داد که میزان جریب و بی‌پویانی در فعالیت آنزیمی در غلظت 
های گوناگون شیر نادران کلسیم، کلسیم و (NaCl) و (CaCl2) انت‌میکروردگان نشان داد که انت‌میکروردگان های آنزیمی را کاهش داد. 
از آنجا که کلر موجود در نمایی کلرید و کلسیم کلرید از راه‌های مارک‌گذاری های انت‌میکروردگان 
پنامی کلرید بیشتر حاصل از پنامی کلرید است تا کل. نتایج دلایل از این دارد که آنزیم 
شیر مهار نمی‌شود و امکان استفاده از آن برای تولید فرآوردهای شیری بدون لازم وجود دارد.

تغییرات متوالی: پنامی‌گر باعث اعمال می‌کند که هیچ نوع تعیین منافعی مرتبط با انتشار این مطالعه تاردید.