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Effect of divalent metal ions on the activity and stability of *β*-galactosidase isolated from *Kluyveromyces lactis*

Adalberto, P.R.[1]; Massabni, A.C.[1]; Carmona, E.C.[2]; Goulart, A.J.[3]; Marques, D.P.[3] Monti, R.[3*]

[1]*Departamento de Química Geral e Inorgânica – Instituto de Química -Araraquara, SP, Brasil.*
[2]*Departamento de Bioquímica e Microbiologia Aplicada – Instituto de Biociências -Rio Claro, SP, Brasil.*
[3]*Departamento de Alimentos e Nutrição – Faculdade de Ciências Farmacêuticas –Araraquara, SP, Brasil. Univ. Estadual Paulista-UNESP.*

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ABSTRACT

In this study, it was demonstrated that β-galactosidase can be deactivated and reactivated with EDTA and divalent metal ions. The enzyme was deactivated after 20 minutes in EDTA solution. Maximal deactivation at the lowest EDTA concentration (10^{-3} mol.L^{-1}) occurred in the presence of Tris-HCl buffer (pH 7.0). The enzyme recovered 50% of its initial activity after 10 minutes at Mg^{2+}concentrations higher than 0.1 mmol.L^{-1}. Experimental concentrations of 0.1 mmol.L^{-1} Mn^{2+} and 1.0 mmol.L^{-1} Co^{2+} were sufficient to reactivate the enzyme to around 300% of the control activity for the Mn^{2+} ion and nearly 100% for the Co^{2+} ion. The enzyme gradually lost its activity when the Co^{2+} concentration was 10^{-2} mol.L^{-1}. Ni^{2+} and Zn^{2+} were unable to restore the catalytic activity. $K_{m\ app}$ and $V_{max\ app}$ were 1.95 ± 0.05 mmol.L^{-1} and 5.40 ± 0.86x10^{-2} mmol.min^{-1}.mg^{-1}, with *o*-NPG as substrate. Optimal temperature and pH were 34°C and 7.5. The half-life ($t_{1/2}$) at 30°C was 17.5 min for the holoenzyme and 11.0 min for the apoenzyme. With respect to pH variation, the apoenzyme proved to be more sensitive than the holoenzyme.

Keywords: β-galactosidase. Divalent metallic ions. Enzyme activity. Stability.

INTRODUCTION

β-galactosidase (EC 3.2.1.23) is the enzyme that catalyzes the hydrolysis of the β-1,4-D-galactoside bond (Pessela et al., 2003). In the food industry, this enzyme is used to remove certain inconvenient components stemming from the production process, as well as to add value to the product. The dairy industry, for instance, produces and uses this enzyme to modify products destined for consumers with lactose intolerance, and to control the crystallization of lactose subjected to low storage and transportation temperatures, as well as enhance the relative sweetness of the product **(**Hoyoux et al., 2001; Todorova-Balvay et al., 2006**;** Ladero et al., 2003**)**. Certain oligosaccharides resulting from the transgalactosylation activity of this enzyme have proved to be efficient probiotics (Ladero et al., 2003**;** Harada et al., 1994). Apart from the processing of dairy products, the food industry also uses β-galactosidase in waste treatment systems (Hoyoux et al**,** 2001; Ladero et al., 2003). It also has applications in clinical trials and stereoselective synthesis. Thus, there is an increasing economic interest in the study of β-galactosidase activity (Hoyoux et al**.,** 2001; Fernandes et al., 2002).

This enzyme is isolated from a wide range of sources, such as bacteria, fungi, actinomycetes, plants, animals and humans (Conchie et al., 1968; Heyworth et al., 1982; Harada et al., 1994; Shaikh et al., 1999; Nakayama et al., 2000; Hoyoux et al., 2001; Fernandes et al., 2002; Lee et al., 2003; Pessela et al., 2003; Todorova-Balvay et al., 2006). As the full use of this enzyme encounters practical limits due to the conditions of use, control of the process and recovery of the enzyme, studies of the effects of metal ions on the activity and immobilization of β-galactosidase may offer solutions to specific needs. In recent years, studies on β-galactosidase from extremophile microorganisms have demonstrated that the enzyme can be stable even in extreme conditions and stabilized derivatives may be obtained by immobilization (Yuan et al., 1994; Fernandes et al., 2002; Ladero et al., 2003**;** Pessela et al., 2004; Adalberto et al., 2006). However, further studies are needed to determine its full potential. Filamentous fungi normally produce acidic extracellular β-galactosidase of varying stability (Shaikh et al., 1999; Adalberto et al., 2006; Todorova-Balvay et al., 2006). The enzyme from *Escherichia coli* is the best studied

Corresponding Author: Rubens Monti - Faculdade de Ciências Farmacêuticas - Araraquara - SP - Brasil - Universidade Estadual Paulista-UNESP - Departamento de Alimentos e Nutrição - Rodovia Araraquara-Jaú-Km1 -CEP.14801-902 - Araraquara - SP - Brasil - telefone: 55 16 3301-6934 email:montiru@fcfar.unesp.br

and its crystallographic structure has been determined (Jacobson et al., 1994). For most of the β-galactosidases studied, divalent metal ions are important in achieving maximal catalytic efficiency and Mg^{2+} is considered the physiological ion (Huber et al., 1979; Martinez-Bilbao et al., 1995; Craig et al., 2000; Sutendra et al., 2007). When the magnesium potential ($pMg=-\log[Mg^{2+}]_{free}$) is below 7, the enzyme becomes more efficient (k_{cat} rises) and has higher affinity for the substrate (K_m falls) (Hoyoux et al., 2001; Sutendra et al., 2007). A number of studies in the literature describe the interaction of β-galactosidase from *E. coli* and *Saccharopolyspora rectivirgula* with divalent ions (Harada et al., 1994; Jacobson et al., 1994; Martinez-Bilbao et al., 1995). However, studies on β-galactosidase isolated from *Kluyveromyces*, of the type reported here are scarce. The divalent ions Mn^{2+}, Mg^{2+} and Ca^{2+} are known to be important to the catalytic activity and stability of many β-galactosidases (Huber et al., 1979; Harada et al., 1994; Sutendra, et al. 2007). β-galactosidase from *E. coli* is a tetramer of identical sub-units (α_4), made up of 1023 amino acid residues, with a molecular mass of 116 kDa, and two bound Mg^{2+} ions per monomer (Jacobson et al., 1994; Yuan et al., 1994; Richard et al., 1995). Craig et al. (2000) demonstrated that there may be two types of galactosidase in commercial extracts, and one of these requires high concentrations of Mg^{2+} during storage for the enzyme to remain active. However, no correlation was found between this need for magnesium and reaction velocity. The substitution of amino acids (Glu-537, Glu-461 and His-540) may promote changes in the activity and inhibitory properties of β-galactosidase (Yuan et al., 1994; Martinez-Bilbao et al., 1995; Roth & Huber, 1996). It is also known that the protein skeleton forms a complex with the metal and must have a second binding site for Mg^{2+}, which is catalytically important. Although this phenomenon is not yet fully understood, it is reasonable to assume that the metal ion participates in the enzymatic catalysis, acting as a Lewis acid. The secondary site, located near the Glu-794 residue, is apparently responsible for the entropic stabilization of the transition state (Sutendra et al., 2007; Martinez-Bilbao et al., 1995). There are studies demonstrating that monovalent cations such as Na^+ and K^+ are activators of the enzyme, whereas Hg^{2+}, Cu^{2+} and Zn^{2+} are inhibitors of the activity of β-galactosidase isolated from *Pseudoalteromonas* (Harada et al., 1994; Roth & Huber, 1996; Fernandes et al., 2002).

Although evolutionary conservation makes β-galactosidase from *E. coli* an efficient model, the enzyme isolated from different sources can exhibit considerable diversity. β-galactosidase from *Saccharopolyspora rectivirgula* is an example of this, as it has eight binding sites for divalent metals per monomer, placed in three classes, according to their affinities; there is a Ca^{2+}-specific site in Class I; a Ca^{2+}-specific site and a Mn^{2+}-specific site in Class II; and four Mn^{2+} and Mg^{2+}-specific sites in Class III. The removal of calcium from the Class I site irreversibly deactivates the enzyme, but not all the enzymes described need Mg^{2+} for activation, although this ion is a stability factor in some enzymes (Harada et al., 1994; Shaikh et al., 1999; Nakayama et al., 2000; Hoyoux et al, 2001; Lee et al., 2003). Acid β-galactosidase from human liver tissue is stimulated and stabilized by the Cl^- ion, in contrast to the inhibitory effect of this ion on other β-galactosidases (Heyworth et al., 1982).

The development of technological processes and devices based on β-galactosidase has captured the attention of researchers and industry alike. While metals are activators of the enzyme and improve its efficiency, their effect on stability determines the best performance of such devices (Shaikh et al., 1999; Hoyoux et al., 2001; Fernandes et al., 2002). The demand for fine tuning of the working conditions for this enzyme makes the presence of metal an important factor (Huber et al., 1979; Harada et al., 1994). The aim of the present study was to investigate the effects of some divalent ions on the activity and chemical and thermal stability of β-galactosidase from *Kluyveromyces lactis,* present in a commercial enzyme extract.

MATERIALS AND METHODS

Reagents: All reagents used: Na_2HPO_4, NaH_2PO_4, $MgCl_2$, $MnCl_2$, $NiCl_2$, $ZnSO_4$, $CoCl_2$, sodium EDTA, Tris-hydroxymethyl-aminomethane and ortho-nitrophenyl-galactoside (*o*-NPG) were of analytical grade and were dissolved in Milli-Q water. The commercial *Kluyveromyces lactis* extract, Lactozym 3000L H PG (lot DKN 08.657), was kindly donated by Novozymes Latin America Ltda. The working buffer Tris/HCl 100 $mmol.L^{-1}$ (pH 7.5) was defined after testing its efficiency in the deactivation of the enzyme. The results were obtained with the enzyme diluted 1000 times in the buffer (final protein concentration 1.04 $mg.mL^{-1}$), in the presence of EDTA (apoenzyme) or its absence (holoenzyme).

Glassware: All glassware involved in the manipulation of the enzyme was washed with milli-Q water and immersed overnight in 1.0 $mol.L^{-1}$ HNO_3 solution. After washing with the acid, the glass was rinsed thoroughly with milli-Q water. Buffers were prepared by mixing suitable volumes of solutions of the acid-base pairs and the pH was confirmed in the aliquots of buffer used (Yuan et al., 1973; Huber et al., 1979; Harada et al., 1994).

Enzyme assay: Enzyme activity was determined by monitoring the hydrolysis of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG), the variation in absorbance at 405nm being followed for 2 min in three independent experiments, by the method proposed by Lederberg & Lederberg (1950), using an Ultrospec 1000 UV/Vis spectrophotometer (Amersham Biosciences). The reaction medium was composed of 50 $mmol.L^{-1}$ sodium phosphate (pH 7.5), 15 $mmol.L^{-1}$ *o*-NPG and 10 $mmol.L^{-1}$ $MgCl_2$. Enzyme deactivation and reactivation tests were conducted by dialysis against EDTA and $MgCl_2$ was excluded from the buffer. The molar absorptivity coefficient was determined in the laboratory through a calibration curve, with *p*-nitrophenol as reference (ε = 13,100 $mol.L^{-1}.cm^{-1}$). One unit of total enzyme activity (U_{total}) of β-galactosidase is defined as the volume of enzyme (in the extract diluted 1000 times) necessary to hydrolyze 1 mmol of *o*-NPG in 1 minute.

Determination of optimal temperature and pH: Optimal temperature was determined by hydrolysis of the chromogenic substrate *o*-NPG in assays conducted in a shaker bath (Dubnoff) at temperatures of 25, 30, 35, 40, 45, 55, and 65°C in the working buffer (50 $mmol.L^{-1}$ sodium

phosphate) at pH 7.5. For the determination of optimal pH, the extract was diluted 1000 times in McIlvaine buffer adjusted to pH between 5.5 and 8.0 (at intervals of 0.5), Tris/HCl buffer (0.10 mol.L^{-1}) adjusted to pH 8.5 and glycine/NaOH adjusted to pH 9.0 to 10.0 (at intervals of 0.5), containing the substrate (15.00 mmol.L^{-1} *o*-NPG) and 1.00 mmol.L^{-1} $MgCl_2$. The enzyme assays for pH were performed at a temperature of 34°C (Shaikh et al., 1999).

Deactivation of the enzyme with EDTA and obtainment of apoenzyme: For the deactivation assay, a volume of 10 μL of enzyme was incubated in 10.00 mL of buffer containing EDTA at concentrations of 0.50, 10^{-2}, 10^{-3} and 10^{-4} mol.L^{-1}. The Tris/HCl (0.10 mol.L^{-1}; pH 7.0) and sodium phosphate (0.050 mol.L^{-1}; pH 7.0) buffers were tested. The free Mg^{2+} content was estimated relative to the total endogenous magnesium in the extract by the concentration of EDTA added (Lee et al., 2003). Activity was determined every ten minutes. Deactivation of the enzyme was analyzed as the logarithmic decrease in residual activity (log %) per minute of incubation time in minutes.

Reactivation of the enzyme with various metals: The apoenzyme was obtained by incubation of the activated enzyme diluted 10 times in the working buffer containing 10^{-3} mol.L^{-1} EDTA, stirred for 1 hour at 4°C. After being diluted 100 times in the working buffer, deactivation was confirmed in the metal-free reaction medium (Lee et al., 2003; Martinez-Bilbao et al., 1995). Volumes of 100 μL of deactivated enzyme were then incubated in 10.00 mL of the Tris/HCl buffer containing the ions Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} at concentrations of 10^{-2}, 10^{-3} and 10^{-4} mol.L^{-1}. The logarithm of the residual activity was analyzed as a function of incubation time.

β-galactosidase stability studies

Stability of the enzyme in relation to pH: One aliquot of the extract was diluted 1:2 (v/v) in the working buffer with EDTA at a concentration of 10^{-3} mol.L^{-1}. A second aliquot of the extract was diluted 1:10 (v/v) in the working buffer without EDTA. After 60 minute under refrigerated stirring (4°C), activity was determined in both samples. The holoenzyme in the working buffer and the apoenzyme in the buffer containing EDTA were diluted 100 times in either McIlvaine buffer (pH 6.0 and 7.5) or Tris/HCl (pH 8.0 to 8.5) or glycine/NaOH (pH 9.0 and 10.5) and incubated at 30°C. Half lives ($t_{½}$ = time necessary for initial activity to be reduced by 50%) were determined from residual activity assays in aliquots removed from the incubation media at programmed time intervals (Nakayama et al., 2000; Pessela et al., 2003). The assay medium contained 15mmol.L^{-1} *o*-NPG and 10^{-2} mol.L^{-1} $MgCl_2$ (Todorova-Balvay et al., 2006).

Thermal stability of the enzyme: To study the thermal stability, the apoenzyme and holoezyme – diluted 1000 times in working buffer with and without EDTA, respectively – were incubated at 30°, 45° and 60°C. At one-minute intervals, aliquots were transferred to an ice bath and residual activity was determined with the *o*-NPG substrate. Logarithms of % residual activity were plotted against incubation time and the apoenzyme and holoenzyme half-lives were determined (Yuan et al., 1994; Martinez-Bilbao et al., 1995).

Kinetic parameters: Kinetic measurements of the extract (diluted 1000 times in the working buffer) were performed at optimal temperature and pH in reaction media containing the substrate *o*-NPG at various concentrations from zero to 32.00 mmol.L^{-1} and 1.0 mmol.L^{-1} $MgCl_2$. Values of the apparent Michaelis-Menten constant ($K_{m\ app}$) and apparent maximal velocity ($V_{max\ app}$) were calculated from Lineweaver-Burk a double reciprocal plot.

RESULTS

β-galactosidase properties: Table 1 displays the kinetic data for *o*-NPG hydrolysis as well as the temperature and pH values at which the enzyme achieved maximal activity. The enzyme was considered mesophilic, with maximal activity at a temperature near 34°C. Optimal pH was slightly basic (approximately 7.5). The diversity of β-galactosidases is considerable and their properties vary from source to source. As examples of thermophilic enzymes, *Rizomucor sp* produces enzymes with an optimal temperature around 60°C, *Thermus sp* produces enzymes with an optimal temperature ranging from 80 to 90°C and *Trichoderma* produces enzymes with an optimal temperature of 65°C (Shaikh et al., 1999; Pessela et al., 2003; Adalberto et al., 2006). These enzymes have optimal pH values ranging from 3.0 to 6.5. Psychrophile organisms produce enzymes at optimal temperatures ranging from 10°C to 26°C and optimal pH values of 8.0 (Hoyoux et al, 2001; Fernandes et al., 2002). Non-microbial sources, such as peaches during maturation, have a β-galactosidase with an optimal temperature of 50°C and optimal pH of 3.0 (Lee et al., 2003).

Table 1. Optimal pH, temperature and kinetic parameters of β-galactosidase from the commercial extract of *Kluyveromyces lactis*, Lactozym 3000 L H PG (lot DKN 08.657).

Property	Value [a]
Optimal temperature	34.0 ± 0.40 °C
Optimal pH	7.50 ± 0.01
$K_{m\ app}$	1.95 ± 0.05 mmol. L^{-1}
$V_{max\ app}$	5.40 ± 0.86 X10^{-2} mmol.min^{-1}.mg$Prot^{-1}$

[a] Values were obtained from graphs plotted with seven data points for the temperature curve, ten points for the pH curve and six points for the kinetic parameters. Data points were means in independent triplicate tests.

The β-galactosidase under study had a typical affinity for *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) and the apparent K_m was 1.95 mmol.L^{-1}. The literature reports lower K_m values for *o*-NPG, such as 0.36 mmol.L^{-1}, for the enzyme isolated from mouse epididymis and 0.16 mmol.L^{-1} for the enzyme isolated from *Pseudoalteromonas*, but values similar to those obtained here are commoner, such as 1.32 mmol.L^{-1} for the enzyme from *Rhizomucor*, 1.65 and 2.66 mmol.L^{-1} for enzymes from *Trichoderma* and 3.0 and 4.0 mmol.L^{-1} for immobilized β-galactosidases from *Termus sp*. The highest K_m value (23 mmol.L^{-1}) has been obtained for the enzyme isolated from the seeds of sweet almonds (Conchie et al., 1968; Fernandes et al., 2002; Pessela et al., 2003; Adalberto et al., 2006).

The apparent maximal velocity ($V_{max\ app}$) of β-galactosidase was estimated at 54 μmol.min^{-1}.mg.prot^{-1}. This may be compared with the velocities exhibited by enzymes from other fungi. Thus, an 82-fold higher velocity is reported for *Rhizomucor* (4.45 mmol.min^{-1}.mg prot^{-1}), while lower maximal *o*-NPG conversion velocities are reported for β-galactosidase from Antarctic psychrophiles (11.7 μmol.min^{-1}.mg prot^{-1}), as well as isozymes from *Trichoderma reesei* that exhibited 0.26 and 0.55 μmol. min^{-1}.mg prot^{-1} (Shaikh et al., 1999; Fernandes et al., 2002; Adalberto et al., 2006).

Deactivation of β-galactosidase with EDTA: Figure 1-A displays the effects of the buffered systems and EDTA concentrations on the deactivation of β-galactosidase isolated from *Kluyveromyces lactis* for the phosphate buffer (0.050 mol.L^{-1}, pH 7.0) and Figure 1-B displays the data for the Tris/HCl buffer (0.10 mol.L^{-1}, pH 7.0). In both buffers, activity declined rapidly, reaching 50% of initial activity in less than five minutes. The decreased in enzyme activity proved to be dependent upon treatment time. For treatments with 0.50 mol.L^{-1} EDTA, a mild activity recovery effect was observed between 15 and 20 minutes in both buffered systems. This effect may be attributed to the pre-equilibrium that is established in the competition of the chelating agent for the metal. At high concentrations of EDTA there is initially a very large shift of this equilibrium towards removal of all the metal from the enzyme. But, after a few minutes, the metal returns to the enzyme, due to the complexation equilibrium between the chelating agent and metal. With the progress of the experiment, the reactivation was soon reversed and maximal deactivation occurred. At all other concentrations of the chelating agent in both systems, deactivation proved to be dependent on time and EDTA concentration. In both buffers, maximal deactivation occurred between 20 and 30 minutes. A slight difference was observed between the systems employed, deactivation under the Tris/HCl buffer being slightly more efficient. The lowest concentration of the chelating agent showing maximal deactivation was 10^{-3} mol.L^{-1} EDTA in Tris/HCl buffer and these conditions were therefore adopted in the following experiments.

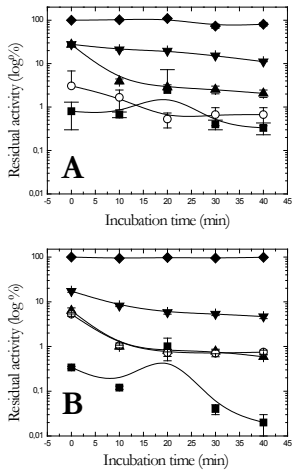

Figure 1. Time-dependent deactivation of β-galactosidase present in the Lactozym 3000 extract, diluted 1000 times in (A) sodium phosphate buffer and (B) Tris/HCl buffer. EDTA –■– 5.0.10^{-1} mol.L^{-1}; –o– 10^{-2} mol.L^{-1}; –▲– 10^{-3} mol.L^{-1}; –▼– 10^{-4} mol.L^{-1}; –◆– control. Assay: 15 mmol.L^{-1} o-NPG, 50 mmol.L^{-1} sodium phosphate buffer pH 7.5, 34°C. Absorbance at 405nm for 2 min.

Activation of ***o***-NPG hydrolysis by divalent metal ions: Having selected the deactivation conditions (tenfold dilution of enzyme in 0.10 mol.L^{-1} Tris/HCl buffer, 10^{-3} mol.L^{-1} EDTA and one hour stirring at 4°C), reactivation assays were performed. Figure 2 shows the results obtained in these assays for the reactivation of β-galactosidase in the Lactozym 3000 commercial extract by Mg^{2+} (A), Mn^{2+} (B), Co^{2+} (C), Ni^{2+} (D) and Zn^{2+} (E). These results demonstrate that the enzyme reverted to approximately 50% of its activity in ten minutes in the presence of free Mg^{2+} at concentrations higher than 0.1 mmol.L^{-1} (Figure 2-A). Reactivation was dependent on the metal concentration. This finding is similar to those reported in the literature on the activation of β-galactosidases from various sources (Harada et al., 1994; Richard et al., 1995; Hoyoux et al., 2001; Lee et al., 2003). It also indicates a strong association between the activity of this enzyme and Mg^{2+} concentration. The results obtained in the assays with Mn^{2+} (Figure 2-B) reveal that this metal was also efficient in reactivating the enzyme. Figure 2-C illustrates the effect of Co^{2+} on the apoenzyme. Once again, an activator effect is obtained with the metal, which appears to be responsible for the recovery of β-galactosidase activity. This effect is less intense than that obtained with Mg^{2+} or Mn^{2+}, as occurs with β-galactosidase from other extracts (Huber et al., 1979; Martinez-Bilbao et al., 1995).

The concentrations of 10^{-4} and 10^{-3}mol.L^{-1} of Mn^{2+} and Co^{2+} were sufficient to reactivate the enzyme to the order of 300% of control for the Mn^{2+} ion and nearly 100% for the Co^{2+} ion. As the concentration of Mn^{2+} or Co^{2+} was increased to 10^{-2}mmol.L^{-1}, the enzyme progressively lost a large part of its activity and became inactive by the end of the experiment. This effect may be explained by the presence of phosphate anions in the reaction medium, responsible for the formation of precipitates under the experiment conditions, which rapidly make the ion unavailable. Thus, although lower concentrations of Mn^{2+} and Co^{2+} activated the enzyme, at higher concentrations the enzyme was reactivated initially, but eventual reversion of the deactivation was not possible.

Figure 2-D displays the activation role of Ni^{2+} on enzyme activity, revealing little or no effect at 10^{-4} and 10^{-3} mol.L^{-1} concentrations, while deactivating the enzyme at a concentration of 10^{-2} mol.L^{-1}. In this respect, the curves for Ni^{2+} and the remaining metals were not equivalent. Figure 2-E displays the deactivation of the enzyme by Zn^{2+} observed in the reactivation tests. There are reports in the literature of an inhibitory effect of Zn^{2+} and Ni^{2+} on other β-galactosidases. Although the effects of high and low concentrations of various metal ions on β-galactosidase activity have been extensively studied, the results of the present study on the reactivation of β-galactosidase present in a commercial extract reveal the importance of examining the particular composition of commercial enzyme extracts, which may have a negative effect on the full reactivation of the enzyme. It should also be stressed that minimal quantities of magnesium, present as an impurity in the reagents, may affect the results of maximal catalytic reactivation of the enzyme, as has also been reported by other authors (Huber et al., 1979).

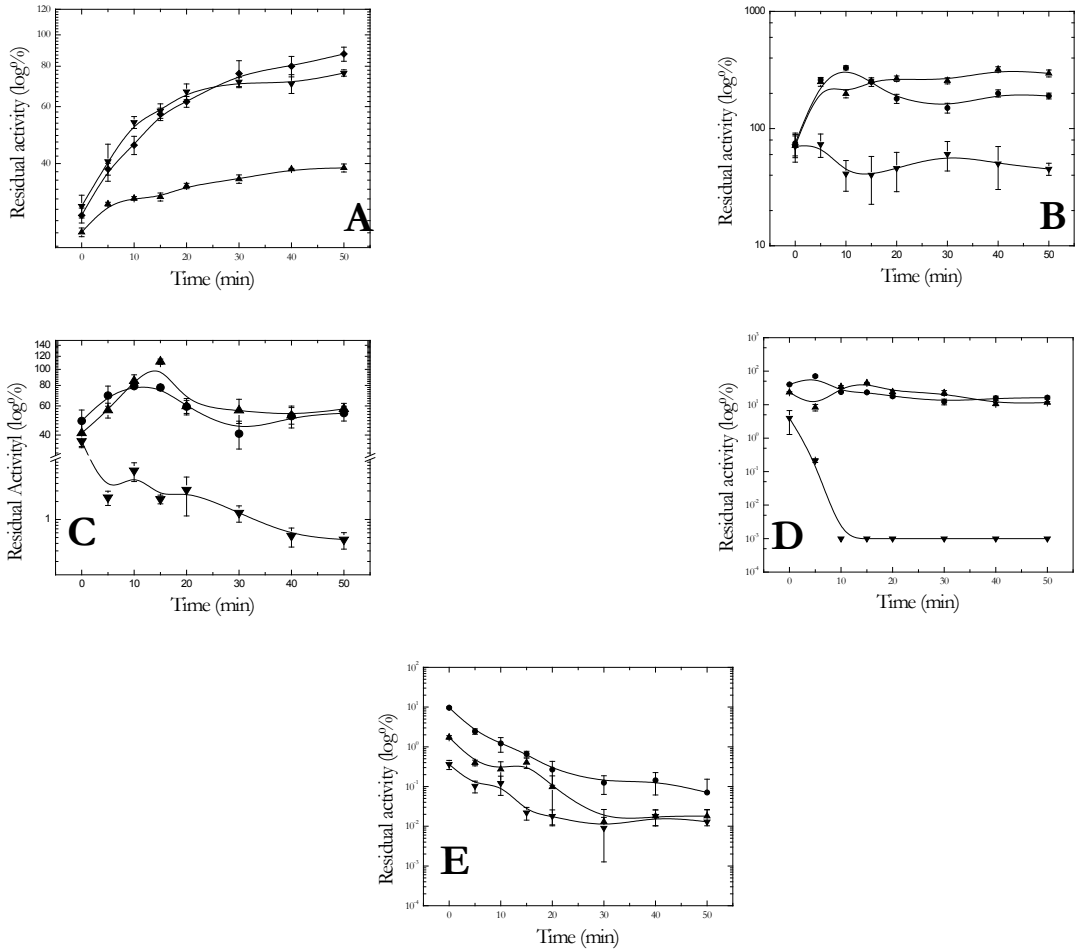

Figure 2. Reactivation of β-galactosidase present in the Lactozym 3000 extract by different metal ions: (A) Mg^{2+}, (B) Mn^{2+}, (C) Co^{2+}, (D) Ni^{2+}, (E) Zn^{2+} in Tris/HCl buffer (10^{-1} mol.L^{-1}, pH 7.0, EDTA 10^{-5} mol.L^{-1}). [Metals]= −●− 10^{-4} mol.L^{-1}; −▲− 10^{-3} mol.L^{-1}; −▼− 10^{-2} mol.L^{-1}.

Thermal and pH stability of the holoenzyme and apoenzyme: Table 2 displays the results for the thermal and pH stability of β-galactosidase. At the temperatures studied, the activity of the holoenzyme fell by half of its maximal value in 17.5 min, when maintained at 30°C, and in less than one minute in the experiments at 45°C and 60°C. Thermal deactivation has been reported to occur in 10 min at 50°C and after 10 min at 60°C for a non-metal-dependent β-galactosidase extracted from peaches (Jacobson et al., 1994). The thermophilic bacteria *Thermus sp* produce a β-galactosidase that is not deactivated before 120 minutes at 80°C (Pessela et al., 2003). Two isozymes have been described for the thermophilic fungus *Trichoderma reesei*, with half-lives of 128 and 118 minutes at 65°C (Adalberto et al., 2006).

The effect of the metal ions on enzyme thermal stability may be observed in the slightly more accentuated decrease in residual activity of the apoenzyme relative to the holoenzyme. Figure 3-A displays the thermal deactivation of both forms, revealing that the apoenzyme reached its half life in 11 min of thermal treatment, while the holoenzyme took 17.5 minutes. This protective effect of the coordinated metal has been described for other β-galactosidases. The multi-metal enzyme from *Saccharopolyspora rectivirgula* has its stability increased when Ca^{2+} and Mn^{2+} occupy their binding sites. An aggregation effect caused by the metal may be involved in the thermoresistance of the enzyme from *Thermus* sp. Bridges spanning between protein units increase the rigidity of the structure, thereby lending greater heat tolerance to the enzyme. Mg^{2+} and, to a greater extent, Mn^{2+} have a protective effect against the thermal deactivation of the enzyme isolated from *E. coli*. The stabilization and aggregation of monomers in this enzyme appear to be related, as resistance to thermal treatment is accompanied by stabilization of the tetramer by the metal (Huber et al., 1979; Harada et al., 1994; Ladero et al., **2003**). The thermostability of acidic β-galactosidase from human liver tissue is dependent on chloride ions and is greatly weakened without this activator ion (Heyworth et al., 1982). The enzyme extract used in the present study proved to be thermosensitive, since at 45°C and 60°C the half-life was reached in less than one minute and little difference was noted between the forms with and without the coordinated metal (results not shown). The enzyme was more sensitive in alkaline than in acid medium (Figure 3-B). At pH values of 9.0 and 10.5, the half-life of the holoenzyme was 9.3 and 9.8 minutes, respectively, whereas at pH 6.0 and 7.5, the half-life was 17.3 and 17.5 minutes (Table 2), respectively. As in the thermal treatment, the apoenzyme proved to be more sensitive to variations in pH than the holoenzyme.

Table 2. Thermal inactivation and thermal stability of β-galactosidase from the commercial extract of *Kluyveromyces lactis*, Lactozym 3000 L H PG (lot DKN 08.657).

	$t_{½}$ (min) [a]						
	Temperature (°C)			pH			
	30	45	60	6.0	7.5	9.0	10.5
Holoenzyme [b]	17.5	<1	<1	17.3	17.5	9.3	9.8
Apoenzyme [c]	11.0	<1	<1	8.7	11.0	7.2	8.2

[a] Time in minutes in which residual activity reached 50% of initial activity, as described in the Materials and Methods section

[b] Obtained by 1000-fold dilution of the enzyme extract in the working buffer

[c] Obtained by 1000-fold dilution of the enzyme extract in the working buffer containing 10^{-4} mol.L^{-1} EDTA.

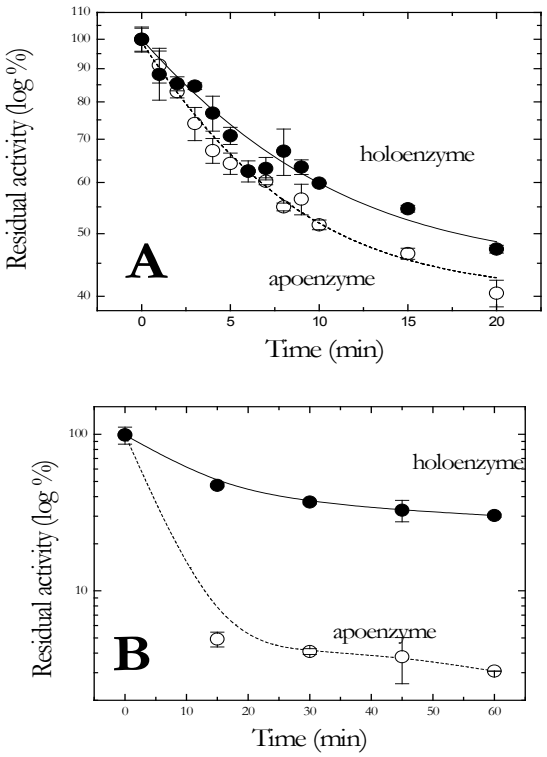

Figure 3. (A) Thermal deactivation at 30°C and (B) chemical deactivation at pH 6.0 of the holoenzyme (−●−) and apoenzyme (−○−) forms of β-galactosidase (Lactozym 3000). The results of the remaining treatments are displayed in Table 2.

DISCUSSION

Optimum temperature and pH of the enzyme β-galactosidase present in the commercial extract were 34°C and pH 7.5, respectively. When this enzyme is extensively dialyzed against EDTA, it loses up to 90% of its activity, which is recovered in the presence of divalent metals. The reversibility of deactivation confirms the effect of the metal ion on its coordination site, while EDTA seems not to be a direct inhibitor of the enzyme (Huber et al., 1979). With the exception of Zn^{2+} and Ni^{2+}, the other metals studied, Mn^{2+}, Mg^{2+}, and Co^{2+}, were able to activate the apoenzyme of β-galactosidase. Among these ions, Mn^{2+} promoted the highest catalytic reactivation, with *o*-NPG as substrate. The apoenzyme activated by Mn^{2+}, in fact, has an activity about 300% higher than when activated by Mg^{2+}. Although Mn^{2+} was more efficient at reactivation than Mg^{2+}, this behavior has not been totally defined, but Mg^{2+} appear to have a regulatory effect on the enzyme activity.

Although this non-specificity for metals is an uncommon property among enzymes, some other metal-dependent enzymes show similar behavior. Phytase from *Bacillus subtilis* is deactivated when the EDTA concentration exceeds that of the metal. Not only is the activity of the enzyme Ca^{2+} dependent, but also its stability is prejudiced in the absence of the ion (Kerovuo et al., 2000). Analysis of secondary structure by circular dichroism (CD) spectroscopy shows that metal stabilize the three-dimensional structure in glutamine synthase from *Azospirillum brasiliense* (Kamnev et al., 2004). Other enzymes, such as the Wistar rat osseous plate alkaline phosphatase, which has the same characteristics, served as a reference for the present work (Ciancaglini et al., 1997).

Enzymatic extract Lactozym 3000 is poorly stable and does not withstand large variations in assay conditions. The susceptibility of the enzyme to pH and heat is greater in the absence of metal ions. In all the treatments carried out, no great decline in residual activity was seen in the holoenzyme. This shows the effect of metal ions on the enzymatic stability. The greater stability of the holoenzyme under changes of pH and temperature is an indication of the role of metal ions in biocatalyst behavior.

Low enzyme stability is a technical problem for the dairy industry, because the hydrolysis of lactose should occur in the period between the packaging and consumption (Ladero et al., 2003). Immobilization of the enzyme allows its reuse and has been used effectively to overcome this problem. Immobilized enzymes may also show biocatalyst improvement and, hence, lower process costs. An example is reported by Mateo et al. (2004), who obtained highly active and stabilized β-galactosidase derivatives, hiding the inhibition site while linking the enzyme to the matrix. Exploration of this technique involves the definition of factors relevant to the catalytic performance. The results presented here contribute to the elucidation of the role of metal ions in the activity and stability of the β-galactosidase present in the Lactozym 3000 extract. These date are relevant to the achievement of stabilized active derivatives of this enzyme of recognized technological importance.

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RESUMO

Efeito de íons metálicos divalentes na atividade e estabilidade da β galactosidase isolada de Kluyveromyces lactis

Este estudo demonstra como a β-galactosidase pode ser desativada e reativada usando EDTA e íons metálicos divalentes. A enzima foi desativada após 20 minutos na presença de EDTA. Desativação máxima para a menor concentração de EDTA (10-3 mol.L-1) ocorreu na presença

do tampão Tris-HCl. A enzima recuperou 50% de sua atividade inicial após 10 minutos na presença de Mg2+ em concentrações superiores a 0,1mmol.L-1. C**oncentrações de 10-4 e 10-3mol.L-1 de Mn2+ e Co2+ foram suficientes para reativar a enzima em 300% comparado ao controle de íons Mn2+ e aproximadamente 100% para íons Co2+.** A enzima perdeu gradualmente a sua atividade quando a concentração foi de 10-2 mol.L-1. Ni2+ e Zn2+ foram incapazes de restabelecer a atividade catalítica. Km app e Vmax app foram 1,95 ± 0,05 mmol.L-1 e 5,40 ± 0,86 x 10-2 mmol.min-1.mg-1. A temperatura e pH ótimos foram 34ºC e 7,5. A meia vida da holoenzima foi de 17,5 min a 30ºC e para a apoenzima foi de 11,0 min a 30ºC. Quanto à variação de pH, a apoenzima provou ser mais sensível que a holoenzima.
Palavras-chave: β-galactosidase. Íons metálicos divalentes. Atividade enzimática. Estabilidade.

REFERENCES

Adalberto PR, Massabni AC, Goulart AJ, Contiero J, Carmona EC, Cardello L, Monti R. Production of β-galactosidase by *Trichoderma reesei* FTKO-39 in wheat bran: partial purification of two isozymes. Appl Biochem Biotechnol. 2006;133(2):163-70.

Ciancaglini P, Pizauro JM, Leone FA. Dependence of divalent metal ions on phosphotransferase activity of osseous plate alkaline phosphatase. J Inorg Biochem. 1997; 66:51-55.

Conchie J, Gelman AL, Levvy GA. Inhibition of glycosidases by aldonolactones of corresponding configuration. The specificity of α-l-arabinosidase. Biochem J. 1968; 106**:**135-40.

Craig DB, Hall T, Goltz DM. *Escherichia coli* α-galactosidase is heterogeneous with respect to a requirement for magnesium. Biometals 2000;13:223-229.

Fernandes S, Geueke B, Delgado O, Coleman R. Beta-galactosidase from a cold-adapted bacterium: purification, characterization and application for lactose hydrolysis. Appl Microbiol Biotechnol. 2002;58:313-21.

Harada M, Inohara M, Nakao M, Nakayama T, Kakudo A, Shibano Y, Amachi T. Divalent metal ion requirements of a thermostable multimetal beta-galactosidase from *Saccharopolyspora rectivirgula*. J Biol Chem. 1994;269(35):22021-26.

Heyworth CM, Neumann EF, Wynn CH. The binding of human liver acid beta-galactosidase to wheat-germ lectin is influenced by aggregation state of the enzyme. Biochem J. 1982;201(3):615-9.

Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, François JM, Baise E, Feller G, Gerday C. Cold-adapted β-galactosidase from the antarctic psychrophile *Pseudoalteromonas haloplanktis.* Appl Environ Microbiol. 2001;67(4):1529-35.

Huber RE, Parfett C, Woulfe-Flanagan H, Thompson DJ. Interaction of divalent cations with beta-galactosidase (*Escherichia coli*). Biochemistry 1979; 19:4090-5.

Jacobson RH, Zhang XJ, DuBose RF, Matthews BW. Three-dimensional structure of beta-galactosidase from *E. coli*. Nature 1994;369:761-6.

Kamnev AA, Antonyuk LP, Smirnova VE, Kulikov LA, Perfiliev YD, Kudelina IA, Kuzmann E, Vértes A. Structural characterization of glutamine synthetase from *Azospirillum brasilense.* Biopolymers 2004;74(1-2):64-8.

Kerovuo J, Lappalainen I, Reinikainen T. The metal dependence of *Bacillus subtilis* phytase. Biochem Biophis Res Comm. 2000;268(2):365-9.

Ladero M, Perez MT, Santos A, Garcia-Ochoa F. Hydrolysis of lactose by free and immobilized beta-galactosidase from Thermus sp. strain T2. Biotechnol Bioeng 2003; 81(2):241-52.

Lederberg J. The beta-D-galactosidase of *Escherichia coli,* Strain K-12. J Bacteriol. 1950; 60(4):381-392.

Lee DH, Kang SG, Suh SG, Byun JK. Purification and characterization of a β-galactosidase from peach (Prunus persica). Mol Cells 2003;15(1):68-74.

Martinez-Bilbao M, Gaunt MT, Huber RE. E461H-beta-galactosidase (Escherichia coli): altered divalent metal specificity and slow but reversible metal inactivation. Biochemistry 1995; 34(41):13437-42.

Mateo C, Monti R, Pessela BCC, Fuentes M, Torres R, Guisán JM, Fernandez-Lafuente R. Immobilization of lactase from *Kluyveromyces lactis* greatly reduces the inhibition promoted by glucose. Full hydrolysis of lactose in milk. Biotechnol Prog. 2004;20:1259-62.

Nakayama T, Goto Y, Hasegawa S, Inohara-Ochiai M, Shibano Y, Ashikari T, Nishino T. Mutational analysis of the role of His452 of Saccharopolyspora rectivirgula beta-galactosidase. J Biosci Bioeng. 2000;90(5):535-9.

Pessela BCC, Torres R, Fuentes M, Mateo C, Munilla R, Vian A, Carrascosa AV, Garcia JL, Guisan JM, Fernandez-Lafuente R. *Selective and mild adsorption of large proteins on lowly activated IMAC matrices. Purification of multimeric thermophilic enzymes expressed in Escherichia coli***.** J Chromatogr. 2004; 1055(1-2):93-8.

Pessela BCC, Vian A, Mateo C, Fernández-Lafuente R, García JL, Guisán JM, Carrascosa AV. Overproduction of *Thermus sp*. Strain T2 beta-galactosidase in *Escherichia coli* and preparation by using tailor-made metal chelate supports. Appl Environ Microbiol. 2003; 69(4):1967-72.

Richard JP, Westerfeld JG, Lin S. Structure-reactivity relationships for β-galactosidase *(Escherichia coli, lac Z).* 2.Reactions of the galactosyl-enzyme intermediate with alcohols and azide ion. Biochemistry 1995;34:11713-24.

Roth NJ, Huber RE. The beta-galactosidase (*E. coli*) reaction is partly facilitated by interactions of His-540 with the C6 hydroxyl of galactose. J Biol Chem. 1996;271:14296-302.

Shaikh SA, Khire JM, Khan MI. Production of galactosidase from thermophilic fungus *Rhizomucor sp*. Biochim Biophys Acta 1999;1472(1-2):239-45.

Sutendra G, Wong S, Fraser ME, Huber RE. Beta-galactosidase (*Escherichia coli*) has a second catalytically important Mg2+ site. Biochem Biophys Res Commun. 2007; 352(2):566-70.

Todorova-Balvay D, Stoilova I, Gargova S, Vijayalakshmi MA. An efficient two step purification and molecular characterization of beta-galactosidases from Aspergillus oryzae. J Mol Recognit. 2006;19(4):299-04.

Villela GG, Bacila M, Tastaldi H. Técnicas e experimentos em Bioquímica. Rio de Janeiro: Guanabara Koogan; 1973. 552p.

Yuan J, Martinez-Bilbao M, Huber RE. Substitutions for Glu-537 of beta-galactosidase from *Escherichia coli* cause large decreases in catalytic activity. Biochem J. 1994; 299:527-31.