Differences in the Hydrolysis of Lactose and Other Substrates by β-D-Galactosidase from *Kluyveromyces lactis*

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ABSTRACT

The hydrolysis of *o*-nitrophenyl galactopyranoside and lactose by β-D-galactosidase from *Kluyveromyces lactis* was enhanced by the addition of Mg^{2+} and Mn^{2+}, but the rates of activation by each metal on both substrates were not the same. The Co^{2+}, Zn^{2+}, and Ni^{2+} activated the *o*-nitrophenyl galactopyranoside-hydrolyzing activity of the enzyme, but these same metals inhibited the lactose-hydrolyzing activity. The addition of Mg^{2+} and EDTA to the assay buffer increased the hydrolysis of *o*-nitrophenyl galactopyranoside and lactose at different rates. The responses of *o*-nitrophenyl galactopyranoside and lactose to the enzyme activity were different as a function of pH. The hydrolyzing activity toward both substrates also was influenced by the concentration of the phosphate in the assay buffer. However, the profile of the enzyme activity toward each substrate was different as a function of concentration. Because the assay of β-galactosidase using *o*-nitrophenyl galactopyranoside is fast and convenient, the estimation of lactose-hydrolyzing activity of the enzyme has frequently been made based on the assay of *o*-nitrophenyl galactopyranoside hydrolysis. As shown in this study, a slight change in the conditions of the assay system and the enzyme application may cause changes in the ability of the enzyme to hydrolyze both lactose and *o*-nitrophenyl galactopyranoside. The change in *o*-nitrophenyl galactopyranoside-hydrolyzing activity is not always consistent with that of the lactose-hydrolyzing activity under the given condition, which may cause an inaccurate estimation of the enzyme activity in the enzyme preparation as well as in actual applications of the enzyme. (**Key words**: β-D-galactosidase, metal effects, substrate specificity)

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Abbreviation key: **LHA** = lactose-hydrolyzing activity, **ONPG** = *o*-nitrophenyl galactopyranoside, **ONPGA** = ONPG-hydrolyzing activity.

INTRODUCTION

The hydrolysis of lactose in dairy products by lactase (β-D-galactosidase; EC 3.2.1.23) can be beneficial in many regards. First, lactose-hydrolyzed dairy products can help lactose malabsorbers, who lack lactase in the intestine and suffer from gastrointestinal disorders such as cramps, flatulence, and diarrhea (9, 14). Second, the hydrolysis of lactose alleviates problems and improves processes for dairy products. The low solubility and lack of sweetness that are often experienced in concentrated milk products and ice cream can be overcome by lactose hydrolysis (19, 20). Cheese that has been manufactured from hydrolyzed milk ripens more quickly than that made from normal milk (4). Third, the use of lactase could also reduce the amount of the lactose in whey which can cause environmental pollution when discharged in large quantities (4, 19, 20).

In the application of lactase, determination of the correct β-D-galactosidase activity is important to obtain the desired final products. The enzyme activity is primarily expressed as color development caused by the hydrolysis of *o*-nitrophenyl galactopyranoside (**ONPG**) to *o*-nitrophenol by the enzyme (17). Because of its relatively fast enzyme turnover rate, good stability in aqueous buffers, and the relative ease by which it can be synthesized or commercially obtained, ONPG is preferred as the substrate for the assay (8). However, a number of reports (1, 5, 16) have suggested discrepancies in the β-D-galactosidase activity to various substrates. The sensitivity of β-D-galactosidase to different substrates has varied. The substrate specificity of the β-D-galactosidase from bacteria and yeasts including *Kluyveromyces lactis* (7, 10), *Escherichia coli* (1, 5), and *Bifidobacterium longum* (16), have also been reported. Rahim and Lee (12) have shown that hydrolyzing activity toward various substrates differs. The conditions of the assay also influence the enzyme activity. Various

metals and ions stimulate or inhibit the activity of β-D-galactosidase (1, 6, 10, 12, 13, 15, 17, 18). The pH, concentration, and type of buffer could change the β-D-galactosidase activity significantly (10, 17). Therefore, the ONPG-hydrolyzing activity (**ONPGA**) of β-D-galactosidase, which is the assay method widely used to estimate the lactose-hydrolyzing activity (**LHA**) of the enzyme in the actual application in dairy products, may not be appropriate to represent the true LHA.

The objectives of this study are to show LHA and ONPGA of a commercial yeast lactase under various conditions that demonstrate the inappropriateness of the assay for ONPG hydrolysis as an estimation of LHA of β-D-galactosidase in actual application.

MATERIALS AND METHODS

Chemicals and Enzyme

All chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO) except for $MgCl_2$ and $MnCl_2$, which were purchased from Mallinkrodt (Paris, KY). A commercially available neutral β-D-galactosidase (lactase; EC 3.2.1.23) from *K. lactis* in 50% glycerin was used for the enzyme preparation.

Assay for ONPG Hydrolysis

The hydrolysis of ONPG was assayed by the method of *Food Chemical Codex* (3). The assay mixture contained 4 ml of preequilibrated 8.29 m*M* of ONPG solution and 1 ml of properly diluted enzyme preparation in PEM buffer (0.2 *M* potassium phosphate, 0.1 m*M* EDTA, and 0.1 m*M* $MgCl_2$; pH 6.5). To study the effects of the metals, they were added to a final concentration of 0 to 400 μmol/g of original enzyme preparation. At the end of incubation (15 min at 37°C), 1 ml of the reaction mixture was added to 1 ml of 10% sodium carbonate solution to terminate the reaction. After the solution stood for 5 min to develop the color, absorbance was measured at 420 nm. One lactase unit is defined as the quantity of enzyme that liberates 1 μmol of ONPG/min.

Lactose Hydrolysis Assay

The enzyme hydrolysis of lactose and the resulting liberation of glucose and galactose were detected by an HPLC method. The mobile phase was 0.01 *N* H_2SO_4, which was filtered through a 0.45-μm filter before use. An IC Pak ion-exclusion (Waters 50Å; 7 μm; Millipore Corporation, Milford, MA) conditioned at 40°C by a column heater was equilibrated with the mobile phase. The carbohydrates were detected by refractive index (Waters 410 differential refractometer; Millipore Corporation); flow rate was 0.9 ml/min. The retention times and the peak area were recorded and analyzed by a Waters 746 data module or Millennium software (Millipore Corporation). Injections were made with a Rheodyne (Cotati, CA) 20-μl injection loop.

The enzyme reaction mixture contained 1 ml of suitable diluted enzyme preparation (7.0 ml of 175 m*M* lactose solution in 2.0 ml of 0.02 *M* potassium phosphate buffer; pH 7.5). The metals were added to a final concentration of 0 to 400 μmol/g of original enzyme preparation. After incubation of this mixture for 15 min at 37°C, the reaction was stopped by boiling for 5 min. The precipitated coagulant was removed by centrifugation (12,000 × *g* for 5 min; Biofuge 13; Baxter, McGaw Park, IL). The supernatant was collected and diluted properly with the mobile phase for HPLC injection. One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1 μmol of lactose into glucose and galactose/min under the assay condition.

Effects of pH and Phosphate Concentration

To study the effects of pH, the reaction mixture was made with the described phosphate buffer for each assay method; each buffer was adjusted to the appropriate pH (6.0 to 8.5). For the reactions in the reconstituted milk, 6.0% nonfat milk solids were dissolved in phosphate buffer at proper pH.

The effect of phosphate concentration was determined by varying the phosphate concentration (0 to 200 m*M*) in the buffer while all other conditions remained the same in each assay method.

RESULTS

Effects of Metals on ONPGA

The effects of metal ions on the ONPGA of β-D-galactosidase were determined. As shown in Figure 1, all of the metals tested acted as activators on ONPGA. The addition of Mg^{2+} and Zn^{2+} caused a steady increase in the activity over the range of metal concentrations tested. The Mg^{2+} and Zn^{2+} enhanced activity 29 and 32%, respectively, at the concentration of 400 μmol/g of enzyme preparation. The Co^{2+} and Mn^{2+} also caused steady increases in ONPGA initially. At 200 μmol/g of enzyme solution, the addition of Co^{2+} and Mn^{2+} resulted in a sudden increase in

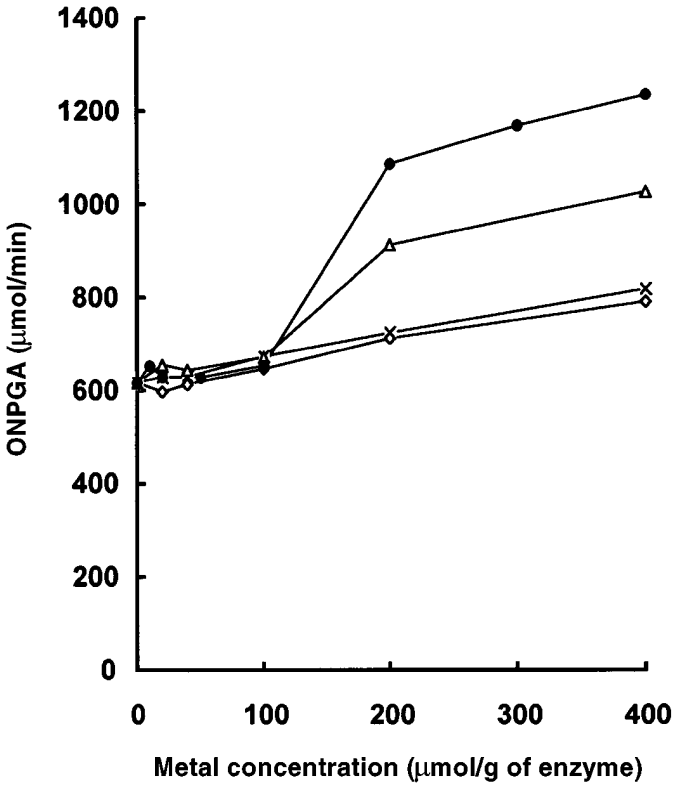

Figure 1. Effects of Co^{2+} (●), Mn^{2+} (△), Mg^{2+} (◊), or Zn^{2+} (×) on *o*-nitrophenyl galactopyranoside-hydrolyzing activity (ONPGA) of β-D-galactosidase.

activity, followed by a steady increase thereafter. The ONPGA was 66 and 100% higher with Co^{2+} and Mn^{2+}, respectively, at 400 μmol/g of enzyme preparation than the initial activity. The Ni^{2+} also activated ONPGA, and 3.6-fold more activity was detected at 400 μmol/g than in the absence of Ni^{2+} (data not shown).

Effects of Metals on LHA

In contrast to ONPGA, the LHA had different patterns (Figure 2). The Zn^{2+} acted as an inhibitor to LHA. At 10 μmol/g, > 50% of LHA was inhibited, and, as addition of Zn^{2+} increased, LHA was inhibited further. The LHA was completely inhibited at 200 μmol/g. Characteristics of activity changes were different with Co^{2+}. Initially, the addition of Co^{2+} enhanced the LHA slightly, followed by the inhibition of the activity at a higher level of Co^{2+}. The LHA was almost completely inhibited at 200 μmol/g of Co^{2+}. A result similar to Co^{2+} addition was obtained with Ni^{2+}, which increased the LHA initially by 20% and then slowly decreased it until the activity was completely inhibited at 300 μmol/g of enzyme preparation. Both Mn^{2+} and Mg^{2+}, however, revealed a different pattern: the LHA was steadily increased to the 200 μmol/g of enzyme solution, which resulted in increases in LHA of 35 and 13% for Mg^{2+} and Mn^{2+}, respectively. However, Mg^{2+} caused a slow decrease in LHA at higher metal concentration.

Effects of pH

The effects of pH on enzyme activity are shown in Figure 3. Both ONPGA and LHA were low pH < 6.0 or pH > 8.5. The pH needed for maximum activity was different for each substrate. The ONPGA was maximal at pH 7.5, but LHA was maximal at pH 6.5.

The effects of Mg^{2+} and EDTA additions were tested over the range of pH because the ONPG assay method based on *Food Chemical Codex* (3) employs PEM buffer with Mg^{2+} and EDTA (Figure 3). In the presence of Mg^{2+} and EDTA in the assay buffer, ONPGA at its highest activity increased 6.4-fold more than the highest activity without Mg^{2+} and EDTA. The pH at maximal activity was shifted from pH 7.5 to 8.0 in the absence of Mg^{2+} and EDTA. The increases in LHA were also observed when Mg^{2+} and EDTA were added to the reaction mixture. However, the degree of the increase at the maximal activity was much smaller (1.5-fold) than that of ONPGA (6.4-

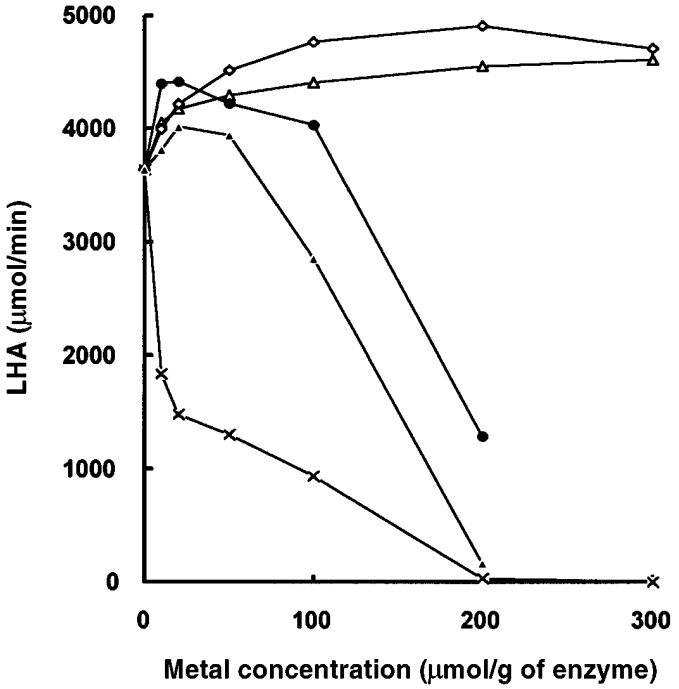

Figure 2. Effects of Co^{2+} (●), Mn^{2+} (△), Mg^{2+} (◊), Ni^{2+} (▲), or Zn^{2+} (×) on lactose-hydrolyzing activity (LHA) of β-D-galactosidase.

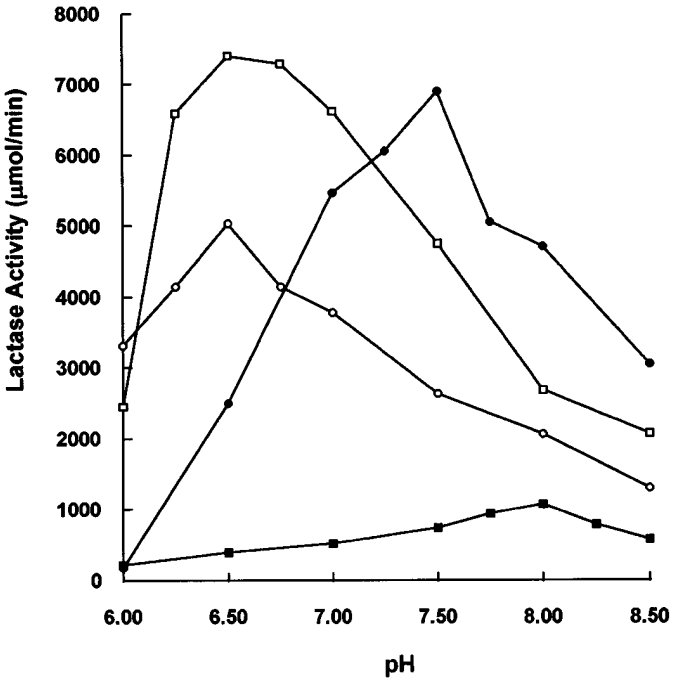

Figure 3. Effects of pH on *o*-nitrophenyl galactopyranoside-hydrolyzing (ONPGA) and lactose-hydrolyzing activity (LHA) of β-D-galactosidase in the presence or absence of Mg^{2+} and EDTA: ONPGA with Mg^{2+} and EDTA (●), ONPGA without Mg^{2+} and EDTA (■), LHA with Mg^{2+} and EDTA (□), and LHA without Mg^{2+} and EDTA (o).

fold). The pH at maximal activity remained at pH 6.5.

The LHA was measured in the reconstituted milk solution in order to simulate the actual application of lactase (Figure 4). When compared with the LHA that was assayed in the phosphate buffer, the LHA in the reconstituted milk was higher and more stable over the pH range tested. The LHA was similarly enhanced in the presence of Mg^{2+} and EDTA in the milk solution. Interestingly, the pH at maximal activity increased from pH 6.5 to 7.0 in the presence of Mg^{2+} and EDTA.

Effects of Potassium Phosphate Concentration

Because a preliminary experiment had shown that the concentration of the potassium phosphate appeared to influence the enzyme activity on both substrates, the concentration effects of potassium phosphate were tested. As shown in Figure 5, both LHA and ONPGA increased up to a certain level of potassium phosphate and decreased thereafter. The LHA was maximum at 40 m*M* of phosphate, and the ONPGA was maximum at 80 m*M*. The maximal LHA at 40 m*M* was 4.3-fold greater than the LHA at 200 m*M* potassium phosphate; ONPGA was only 1.8-fold greater at the maximum concentration of 80 m*M* than at 200 m*M*.

DISCUSSION

The use of lactase in dairy products can benefit lactose malabsorbers (9, 14) and the dairy industry (4, 19, 20). In most cases, lactase activity is represented by the assays using ONPG as a substrate for convenience (8, 17). This study showed that slight modifications in assay conditions and in application of the enzyme influenced ONPGA and that these changes were not always consistent with the changes in LHA.

All of the metals examined activated ONPGA under tested conditions. Only Mg^{2+} and Mn^{2+} activated LHA slightly, but other metals (Co^{2+}, Ni^{2+}, and Zn^{2+}) inhibited the LHA partially or completely. Mahoney and Whitaker (10) reported that lactase from *K. fragilis* was activated by Mn^{2+}, Mg^{2+}, Co^{2+} Zn^{2+} in potassium phosphate buffer, but not in Tris buffer, which indicated that the same metals can have a

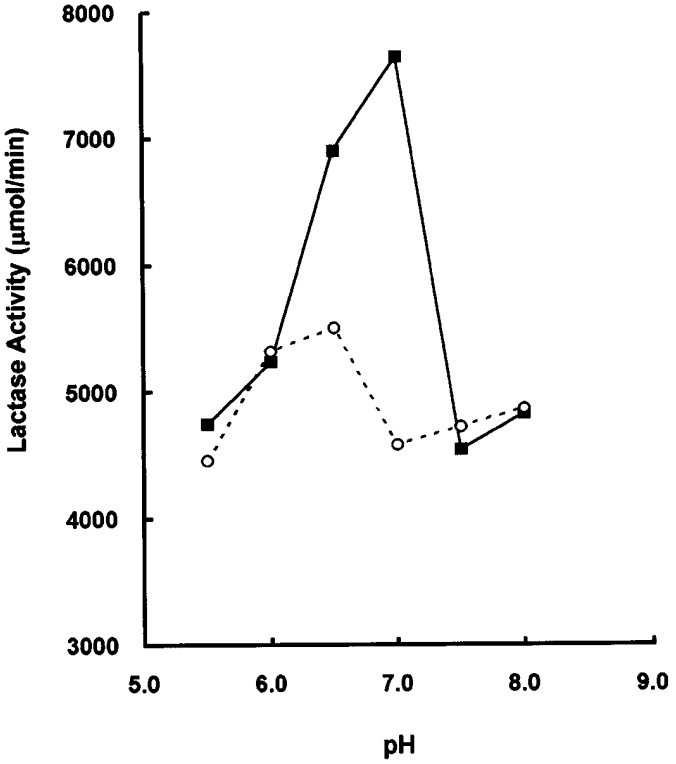

Figure 4. Effects of pH in the reconstituted milk on lactose-hydrolyzing activity (LHA) of β-D-galactosidase in the presence or absence of Mg^{2+} and EDTA: LHA in the absence of Mg^{2+} and EDTA (o), and LHA in the presence of Mg^{2+} and EDTA (■).

different effect depending on conditions. However, those researchers assayed the enzyme activity using ONPG and did not compare the metal effects directly on lactose or other substrates. Some reports (7, 10, 18) showed the substrate specificity of lactase from *Kluyveromyces* and *Saccharomyces* spp., but none revealed the possible contradictory effects of metal addition on enzyme activity toward lactose and other substrates. The effect of various metals on β-D-galactosidase from *E. coli* was studied by Cohn and Monod (2). All metals enhanced ONPGA, but the same metal ions, including Mn^{2+}, inhibited LHA to various degrees; Mg^{2+} was an exception. The addition of Cu^{2+} and Zn^{2+} inactivated LHA completely at 1 m*M*. Even though the results of Cohn and Monod (2) were similar to those of this study, the results may not be comparable because 1) those researchers tested only one concentration, and 2) the enzyme came from different origin. Nakano et al. (11) stated that enzymes from different origins showed different activity toward the same substrate.

The ONPG assay method based on *Food Chemical Codex* (3) used PEM buffer. The addition of Mg^{2+} and EDTA in the buffer increased both ONPGA and LHA, but ONPGA increased much more than LHA (Figure 3). These results indicated that the degrees of activation or inhibition of ONPGA and LHA are different under the same condition even if both activities are activated or inhibited concurrently. The pH of maximal ONPGA shifted from 8.0 to 7.5 with the addition of Mg^{2+} and EDTA, but maximal pH for LHA remained unchanged. In a review, Wallenfels and Weil (17) indicated that the pH dependence of lactase activity (ONPGA) is strongly influenced by divalent ions and suggested the presence of two active groups that are protonated at different pH. However, their theory (17) cannot explain why the maximal pH of LHA remained the same in the presence of Mg^{2+} and EDTA.

Other evidence of differences in activity was the response to the concentration of potassium phosphate in the buffer. The ONPGA and LHA were maximal at different phosphate concentrations. Wendorff and Amundson (18) showed activation of lactase from *Saccharomyces fragilis* by K^+ but only in the presence of Mn^{2+} as a cofactor for the enzyme. However, the activation profile of the enzyme in response to the change in potassium phosphate concentration toward different substrates has not been reported.

Numerous investigations on β-galactosidase have been performed, and many modifications of assay methods with ONPG or other substrates have been made (17). Those investigations have employed various concentrations of phosphate and other types of buffer for the assay for ONPGA, and various amounts and types of metal ions were added, especially Mg^{2+} and Mn^{2+}, to the assay system. Some reports (10, 17) have suggested the stimulation or activation of β-D-galactosidase in modified conditions to improve lactase performance. However, those reports estimated LHA based on ONPGA that was assayed under greatly modified conditions. As shown in this study, slight changes in the concentrations of metal and buffer may change the ONPGA of β-D-galactosidase, and changes in the ONPGA do not always coincide with the changes in LHA.

Many studies have been carried out to determine the effects of various conditions on the β-D-galactosidase activity toward different substrates. Few of the reports, however, have emphasized the direct comparison between LHA and hydrolyzing activity of other substrates, including ONPG, under the same conditions. Little is found in the literature about the importance of the differences in hydrolyzing activity of substrate in actual application of the enzyme in the industry and academia.

The use and the study of commercial lactase to benefit lactose malabsorbers and the dairy industry are increasing (11). In most cases, lactase activity is represented by the assay using ONPG as a substrate. Because the enzyme may have different hydrolyzing activity toward different substrate under certain conditions, it may not be appropriate to use an activity unit based on the ONPGA as an estimation of LHA

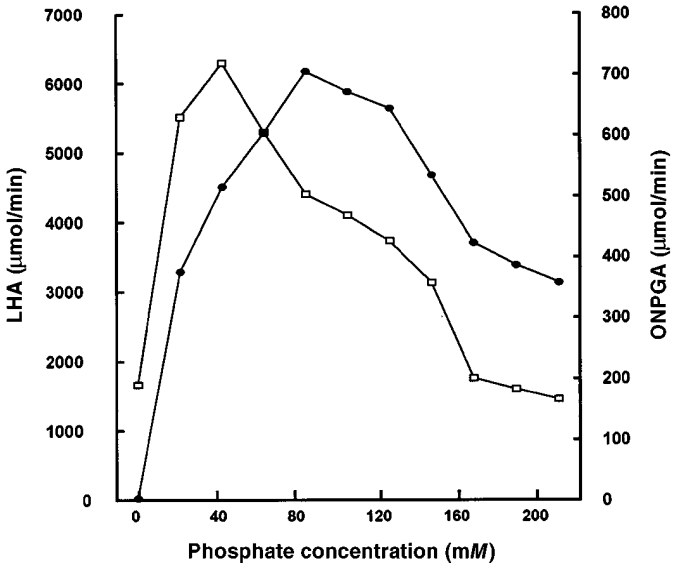

Figure 5. Effects of phosphate concentration in the buffer on lactose-hydrolyzing activity (LHA; □) and *o*-nitrophenyl galactopyranoside-hydrolyzing activity (ONPGA; ●) of β-D-galactosidase.

under identical conditions or actual application.

To overcome the discrepancies in the enzyme activities toward different substrates, the degree of lactose hydrolysis in the actual application has to be measured to determine the application level. In addition, certain minerals and conditions could be optimized to obtain the best enzyme performance without interfering with the product formulation and processes, which may not be predicted properly by the ONPG assay alone.

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