Hydrolysis of lactose by β-galactosidase from *Kluyveromyces fragilis*: characterization of the enzyme

Graciette Matioli*[1], Flávio Farias de Moraes[2] and Gisella Maria Zanin[2]

*[1]Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. [2]Departamento de Engenharia Química, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. *Author for correspondence. e-mail: gmatioli@uem.br*

ABSTRACT. The β-galactosidase enzyme from *Kluyveromyces fragilis* was characterized in the soluble form using lactose 5% w/v found in skimmed powdered milk as substrate. Enzyme diluted 50 times hydrolyzed the lactose in batch reactor of 50 mL capacity. Enzyme activity and its activation energy were determined as a function of temperature and pH. Temperature ranged from 20 to 55ºC and pH from 5.5 to 8.0. Activation energy was 9.50 kcal/mol. The energy of deactivation was 33.74 kcal/mol. Although the enzyme presented a high specific activity at 45ºC and pH 6.5 (3.312 U/mg protein), values indicate that the best use of the enzymatic activity occur at 40ºC or below, with half-life higher than 12 hours. The activation energy increased proportionally to pH increase. Therefore, the activation energy depends on pH and varies according to the origin of the enzyme.

Key words: β-galactosidase, lactase, lactose, *Kluyveromyces fragilis,* activation energy, energy of deactivation.

RESUMO. Hidrólise da lactose pela β-galactosidase de *Kluyveromyces* fragilis: caracterização da enzima. A enzima β-galactosidase de *Kluyveromyces fragilis* foi caracterizada na forma solúvel, utilizando como substrato, lactose 5% p/v presente no leite em pó desengordurado. A enzima, diluída 50 vezes, hidrolisou a lactose em reator batelada de 50 ml de capacidade. A atividade da enzima e sua energia de ativação foram determinadas em função da temperatura e pH. A faixa de temperatura analisada foi de 20 a 55ºC e de pH de 5,5 a 8,0. A energia de ativação foi de 9,50 kcal/mol. A energia de desativação foi de 33,74 kcal/mol. Embora a enzima tenha apresentado uma atividade específica alta a 45ºC e pH 6,5 (3,312 U/mg proteína), os valores obtidos indicam que o melhor aproveitamento da atividade enzimática se dá a 40ºC ou abaixo, com um tempo de meia-vida superior a 12 horas. A energia de ativação aumentou proporcionalmente com o aumento de pH. Portanto, a energia de ativação depende diretamente do pH da solução e varia com a origem da enzima.

Palavras-chave: β-galactosidase; lactase; lactose; *Kluyveromyces fragilis*; energia de ativação; energia de desativação.

Lactose, a major component of milk and whey, used in food products, is somewhat limited due to its low solubility and indigestibility in many individuals. For this reason, lactose is often hydrolyzed before use.

The cheese industry produces large amounts of lactose in the form of cheese whey and whey permeate, generating approximately 27 million tonnes/yr in the U.S. alone. Many uses have been found for whey and lactose, even in formulae for infants; bakery, dairy and confectionery products; animal food; and feedstocks for lactose derivatives and several industrial fermentations. Still, demand is insufficient for all available whey lactose. The result is a low market value for lactose; almost half of the whey produced each year remains unused, which eventually becomes a significant waste disposal problem. Justifiable environmental concerns over dumping this waste with high biological oxygen demand have ended that practice, leaving dairies with enormous amounts of whey (Yang and Silva, 1995).

Lactose maldigestion, which is generally attributable to a genetically predetermined reduction in β-galactosidase (lactase) activity in the small intestine according to age and sometimes after weaning, is a frequent cause of gastrointestinal symptoms in pediatric patients. Abdominal pain, flatulence, or diarrhea may result from the fermentation of undigested lactose by colonic

bacteria with production of H_2, CH_4, CO_2, and short-chain organic acids. Children and adolescents represent a major segment of milk-drinking population, and reduction or elimination of milk and dairy products from the diet of children with lactose maldigestion may compromise their intake of protein, riboflavin and calcium. Accordingly, alternatives to milk elimination have been developed. Currently available validated approaches include the use of microbially derived β-galactosidase added to milk to prehydrolyze the lactose or taken when foods containing lactose are ingested. Powdered fermented milk that retains β-galactosidase activity also reduces lactose maldigestion in children (Noh and Gilliland, 1994; Montes *et al.,* 1995; Jiang *et al.,* 1996).

Lactose-intolerant individuals tolerate fermented milks better than fresh milk with the equivalent amount of lactose. A generally accepted explanation for this is that the lactose-hydrolyzing enzyme (β-galactosidase) contained within the microbial cells of the yogurt substitutes for the paucity of β-galactosidase in the small bowel mucosa of lactose-intolerant individuals. It has been proposed that improved fermented milk products could be derived by using strains of bacteria that possess high β-galactosidase activity at acidic pH. Lactose hydrolysis in the proximal intestinal tract would avoid the fermentation of lactose by the normal microflora inhabiting the distal intestinal tract, which causes the symptoms of lactose intolerance (Kotz *et al.*, 1994; Burton and Tannock, 1997).

Another approach to the management of lactose digestion is the consumption of unfermented milk containing live lactic acid bacteria like *Lactobacillus acidophilus* and *Bifidobacterium longum* (Jiang *et al.,* 1996; Mustapha *et al.*, 1997). The β-galactosidase activity of *L. acidophilus* can be increased in the presence of bile. Bile apparently increases the permeability of cell membranes of *L. acidophilus*, allowing more substrate to enter and be hydrolyzed (Noh and Gilliland, 1994; Valdez *et al.*, 1997).

The hydrolysis of lactose in dairy products by β-galactosidase may be beneficial from another aspect. Lactose hydrolysis 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 may be overcome by lactose hydrolysis. Cheese that has been manufactured from hydrolyzed milk ripens more quickly than that made from normal milk (Kim *et al.*, 1997).

In the application of lactase, determination of the correct β-galactosidase activity is important to obtain the desired final products. The aim of current research is the characterization of the β-galactosidase from *Kluyveromyces fragilis* using skimmed powdered milk as substrate.

Material and methods

Enzyme. The enzyme β-galactosidase from *Kluyveromyces fragilis* (LACTOZYM 3000 LAU/L) was supplied by the NOVO NORDISK (Copenhagen, Denmark). The best concentration of the enzyme, determined by the Lowry method (Lowry *et al.,* 1951), was 16.2189 mg of protein/mL, that corresponds to a dilution of 1:50.

Substrate. Solution of lactose 5% w/v in the skimmed powdered milk from MOLICO (Nestlé) has the following medium constitution: lipid 1.0%, protein 36.0%, lactose 52.0%, salts minerals 8.0% and water 3.0%.

Buffer solution. The solution was prepared with deionized water, 0.01% of sodium azide as preservative, in such a way as to supply the chemical components in the concentration of minerals found naturally in the milk, according to recommendations of the NOVO (1979). The pH was adjusted with NaOH 4 N for pH 6.5; about 10% v/v of that preparation was used in each assay. The adjustment of pH of the solution below 6.5 was achieved by buffer disodium phosphate-citric acid 0.1 M. This solution was prepared according to method by Mcllvaine (Morita and Assumpção, 1972).

β-galactosidase activity assay. One unit of activity (U) corresponds to the amount of enzyme that liberates a micromol of glucose per minute in reaction conditions. The activity assay conditions consisted of a lactose solution 5% w/v present in skimmed powdered milk, at temperature 45°C and pH 6.5. The specific activity was obtained by dividing the activity by the protein mass used in the assay. The buffer-diluted enzyme solution (1:50) was prepared and 1.0 mL of this solution was added in a glass bath reactor, agitated and the temperature stabilized in warm-bath, containing 50 mL of the substrate. Samples of 0.5 mL were removed at regular intervals, until completing a total of 30 minutes, and added to tubes containing 2.5 ml of water. Inactivation of enzyme was accomplished by heating the tube in boiling water for 10 minutes. The reaction time and enzyme dilution were selected in such a way as to have a linear relationship between the formed glucose and time, seeking the reduction of the effect of the inhibition of the reaction products, according to criteria established by the method of initial velocities (Dixon and Webb,

1979). The glucose formed in the reaction was determined by the GOD-PAP spectrophotometric method, in which the enzymatic determination of the glucose with the enzyme peroxidase is done (Barham and Trinder, 1972).

Enzymatic activity as a function of temperature and pH. In the glucose formation test the substrate solution was prepared at the following pH values: 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The tests were performed, as in the case of the enzymatic activity assay, at 45ºC. The influence of the temperature on the activity of the enzyme was determined at the temperature range of 20 to 55ºC with an interval of 5ºC. Tests were performed at pH 6.5, as in the enzymatic activity assay. Glucose produced was determined as in section β-galactosidase activity assay mentioned above.

β-galactosidase thermal stability. The residual activity of the β-galactosidase LACTOZYM 3000 L was determined by initial velocities method (Dixon and Webb, 1979). 1.0 mL of the enzyme was incubated in 100 mL of the lactose solution 5% w/v, pH 6.5, during 240 minutes at temperatures from 20 to 55ºC. Diluted enzyme solution was maintained in a selected temperature and every 40 minutes an aliquot of 1.0 mL was taken from it and added to glass bath reactor containing 50 mL of the lactose solution 5% w/v, pH 6.5, 45ºC. Samples of 0.5 mL were removed every 5 minutes, for 30 minutes, and added in tubes with distilled water. The inactivation of the enzyme was accomplished by heating the tubes in boiling water for 10 minutes. This procedure was repeated up to 240 minutes of incubation of enzyme at each selected temperature (from 20 to 55ºC). Tubes from the residual activity test were maintained at 4ºC for later determination of the produced glucose by spectrophotometric method indicated in the section β-galactosidase activity assay.

Results and discussion

Characterization of β-galactosidase

Activity of the enzyme as a function of pH and temperature. Results of the assay of specific activity (Ae) as a function of pH and temperature for the enzyme β-galactosidase from *Kluyveromyces fragilis* are demonstrated in Figure 1. The enzyme presented maximum activity in pH 6.5, in practically every range of analyzed temperature (20 to 55ºC), except for 50 and 55ºC, whose maxim activity occurred in the pH 7.0. Temperature of maximum specific activity of enzyme in practically all values of pH (5.5 to 8.0) was 45ºC, reaching its maximum in pH 6.5 (3.12 U/mg of protein). In this pH value at 55ºC it was 9.2 times less (0.34 U/mg of protein). Temperature values and pH obtained in this research agree with those found in the literature (Mahoney and Whitaker, 1977; Nijpels, 1982; Hernandez and Asenjo, 1982).

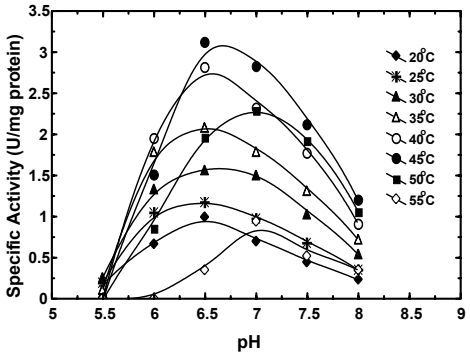

Figure 1. Specific activity as a function of pH and temperature for enzyme β-galactosidase from *Kluyveromyces fragilis*. Substrate: lactose solution 5% w/v

Energy of enzyme activation. Effect of temperature on an enzymatic reaction may be analyzed by the Arrhenius type equation. The equation adjusted to the experimental points of Figure 1 and 2 allowed the determination of the activation energy (cal/mol) and enzymatic activity for the reaction of hydrolysis of lactose 5% w/v and glucose production, in pH values 5.5 to 8.0. Result consists of the following equations, plotted on Figure 2:

pH 5.5: $Ae = 8.6379 \times 10^4 \exp(-76781/RT)$
pH 6.0: $Ae = 6.4920 \times 10^5 \exp(-81729/RT)$
pH 6.5: $Ae = 1.1045 \times 10^7 \exp(-94956/RT)$
pH 7.0: $Ae = 1.0150 \times 10^8 \exp(-109697/RT)$
pH 7.5: $Ae = 1.5784 \times 10^8 \exp(-114162/RT)$
pH 8.0: $Ae = 3.4189 \times 10^8 \exp(-122707/RT)$

where R is the ideal gas constant (1.987 cal/(mol x K) and T is the absolute temperature in Kelvin.

The activation energy of the reaction of hydrolysis of lactose 5% w/v and pH 6.5 was 9.50 kcal/mol for temperatures ranging from 20 to 55ºC.

Figure 3 presents activation energy of the reaction of hydrolysis of the lactose for enzyme β-galactosidase of *Kluyveromyces fragilis* as a function of the pH. It shows that activation energy depends directly on the pH of solution. Since it varies according to the origin of the enzyme, it could be represented by the equation:

$$Ea = -3.091 + 1.9297\ pH$$

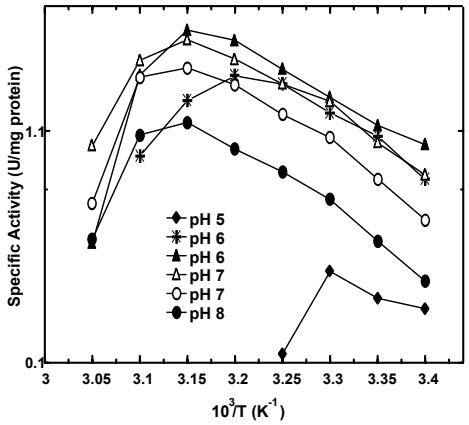

Figure 2. Arrhenius plot of the specific activity for glucose production as a function of the inverse of the absolute temperature for the β-galactosidase from *Kluyveromyces fragilis*. Substrate: lactose solution 5% w/v

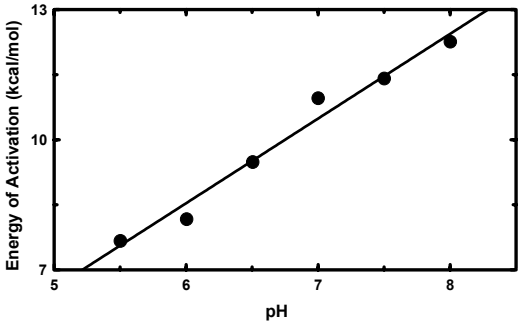

Figure 3. Activation energy of hydrolysis reaction of lactose as a function of pH. Enzyme: β-galactosidase from *Kluyveromyces fragilis*. Substrate: lactose solution 5% w/v

Thermal stability of the enzyme. Figure 4 shows the residual specific activity for glucose production as a function of time for β-galactosidase from Kluyveromyces *fragilis*. Although the data presented in the Figure 1 show that the enzyme has a high specific activity at 45ºC and pH 6.0, its thermal stability in these conditions is relatively low. Its use above 4 hours becomes unfeasible. While at 45ºC and pH 6.0 the enzyme showed a value of 3.12 U/mg of protein, as previously verified, the specific activity at 40ºC and same pH gave value 2.81 U/mg of protein, that is, 1.1 times less than at 45ºC. It is thus advisable to maintain a temperature of 40ºC, in which the enzyme shows a relatively high activity and good thermal stability. Comparable results were found by Mahoney and Whitaker (1977) and Hernandez and Asenjo (1982).

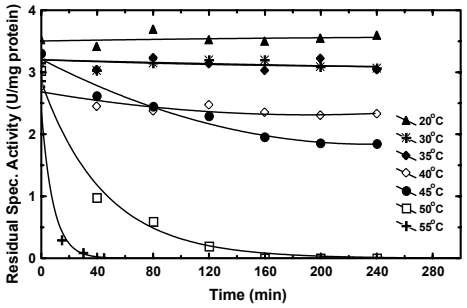

Figure 4. Residual specific activity for glucose production as a function of time for the β-galactosidase from *Kluyveromyces fragilis*. Conditions: substrate consists of lactose solution 5% w/v and pH 6.5

Energy of thermal deactivation of the enzyme. It has been reported in Figure 4 that the β-galactosidase didn't present thermal deactivation at temperatures ranging from 20 to 35ºC, with an activity retention after 240 minutes of incubation of 100%; at 45ºC it reached 55,8% of the initial activity. At 50ºC and above, the enzyme presented accentuated thermal inactivation, mainly at 55ºC; however, after 30 minutes of assay no more activity was observed.

The half-life of β-galactosidase was calculated by the exponential model (Matioli, 1997; Zanin and Moraes, 1998). In the case of enzyme incubated in a solution of lactose 5% w/v, pH 6.5, the half-life was higher than 12 hours for temperatures lower than 40ºC; it dropped to just 6 minutes at 55ºC. For long reaction periods these results delimit the best use of the enzymatic activity to temperatures lower than 40ºC.

It is usually assumed that the kinetics of enzyme thermal deactivation is first with regard to concentration of active enzyme, and that the coefficient of thermal inactivation is a function of the temperature, as given by the Arrhenius law. The slope of the adjusted straight line that correlates the natural logarithm of the coefficient of thermal inactivation with the inverse of the absolute temperature is the energy of thermal deactivation (Matioli, 1997; Zanin and Moraes, 1998). This graph is shown in the Figure 5 and the energy of deactivation obtained was 33.74 kcal/mol. Value is about 1.7 times higher than that found by Mahoney and Whitaker (1977). However, in this case a different substrate, the *o*-nitrophenyl-β-D-galactopyranoside, was used.

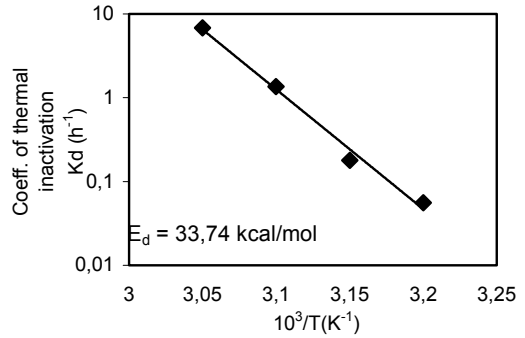

Figure 5. Arrhenius plot of the coefficient of thermal inactivation as a function of the inverse of the absolute temperature for the β-galactosidase from *Kluyveromyces fragilis*. Conditions: substrate consists of lactose solution 5% w/v and pH 6.5

The β-galactosidase from *Kluyveromyces fragilis* is less stable than Novo Nordisk (Copenhagen, Denmark) amyloglucosidade with a deactivation energy of 50.6 kcal/mol (Zanin and Moraes, 1998).

Acknowledgements

The authors would like to thank NOVO NORDISK for the supply of the enzyme used in this research. Thanks are also due to CNPq, CAPES, UEM for their financial support.

References

BARHAM, D.; TRINDER, P. Improved color reagent for the determination of blood glucose by the oxidase system. *Analyst*, London, v. 97, p.142-145, 1972.

BURTON, J. P.; TANNOCK, G. W. Properties of porcine and yogurt lactobacilli in relation to lactose intolerance. *J. Dairy Sci.,* Champaing, v. 80, p.2318-2324, 1997.

DIXON, M.; WEBB, E. C. *Enzymes*. 3. ed. London: Longman Group Limited, 1979.

HERNANDEZ, R.; ASENJO, J. A Production and characterization of an enzymatic hydrolysate of skim milk lactose and proteins. *J. Food Sci.*, Chicago, v. 47, p.1895-1898, 1982.

JIANG, T. *et al.* Improvement of lactose digestion by humans by ingestion of unfermented milk containing *Biffidobacterium longum. J. Dairy Sci.,* Champaing, v.79, p.750-757, 1996.

KIM, S.H. *et al.* Differences in the hydrolysis of lactose and other substrates by β-D-galactosidase from *Kluyveromyces lactis*. *J. Dairy Sci.,* Champaing, v. 80, 2264-2269, 1997.

KOTZ, C.M. *et al.* Factors affecting the ability of a high β-galactosidase yogurt to enhance lactose absorption. *J. Dairy Sci.,* Champaing, v. 77, p.3538-3544, 1994.

LOWRY, O.H. *et al.* Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* Baltimore, v. 193**,** p. 265-275, 1951.

MAHONEY, R. R.; WHITAKER, J. R. Stability and enzymatic properties of beta-galactosidase the *Kluyveromyces fragilis*. *J. Food Sci.*, Chicago, v. 42, p.327-350, 1977.

MAHONEY, R. R., WHITAKER, J. R. Purification and physicochemical properties of beta-galactosidase the *Kluyveromyces fragilis*. *J. Food Sci.*, Chicago, v. 43, p.584-591, 1978.

MATIOLI, G. *Seleção de microrganismo e caracterização de sua enzima ciclodextrina glicosiltranferase.* 1997. Tese (Doutorado em Bioquímica) - Universidade Federal do Paraná, Curitiba, 1997.

MONTES, R.G. *et al.* Effects of milks inoculated with *Lactobacillus acidophilus* or a yogurt starter culture in lactose-maldigesting children. *J. Dairy Sci.,* Champaing, v. 78, p.1657-1664, 1995.

MORITA, T.; ASSUMPÇÃO, R. M. V. Manual de soluções reagentes e solventes – padronização – preparação – purificação. São Paulo: Edgard Blucher Ltda., 1972.

MUSTAPHA, A. *et al.* Improvement of lactose digestion by humans following ingestion of unfermented acidophilus milk: influence of bile sensitivity, lactose transport, and acid tolerance of *Lactobacillus acidophilus. J. Dairy Sci.,* Champaing, v. 80, p.1537-1545, 1997.

NIJPELS, H. H. *Developments in food carbohydrate.* London: Applied Science Publishers Ltd., 1982.

NOH, D. O.; GILLILAND, S. E. Influence of bile on β-galactosidase activity of component species of yogurt starter cultures. *J. Dairy Sci.,* Champaing, v. 77, p.3522-3537, 1994.

NOVO *Application of yeast lactases: a review*. Bagsvaerd: Novo Nordisk. 1979. 28 f. Novo's file number A 5489.

VALDEZ, G. F. *et al.* Influence of bile on β-galactosidase activity and cell viability of *Lactobacillus reuteri* when subjected to freeze-drying. *J. Dairy Sci.,* Champaing, v. 80, p.1955-1958, 1997.

YANG, S. T.; SILVA, M. Novel products and new technologies for use of a familiar carbohydrate, milk lactose. *J. Dairy Sci.*, Champaing, v. 78, p.2541-2562, 1995.

ZANIN, G. M.; MORAES, F. F. Thermal stability and energy of deactivation of free and immobilized amyloglucosidase in the saccharification of liquefied cassava starch. *Appl. Biochem. Biotechnol.*, New Jersey, v. 70-72**,** p.383-394, 1998.

Received on March 06, 2001.
Accepted on April 23, 2001.