ISSN 1330-9862
(FTB-2952)

original scientific paper

Kinetics and Thermal Properties of Crude and Purified β-Galactosidase with Potential for the Production of Galactooligosaccharides

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Received: October 19, 2011
Accepted: September 6, 2012

Summary

β-Galactosidase is an enzyme that catalyzes the hydrolysis of lactose. It has potential importance due to various applications in the food and dairy industries, involving lactose-reduced ingredients. The properties of two β-galactosidase enzymes, crude and purified, from different sources, *Kluyveromyces marxianus* CCT 7082 and *Kluyveromyces marxianus* ATCC 16045, were analyzed. The pH and temperature optima, deactivation energy, thermal stability and kinetic and thermodynamic parameters were determined, as well as the ability to hydrolyze lactose and produce galactooligosaccharides. Purification process improved the properties of the enzymes, and the results showed that purified enzymes from both strains had a higher optimum temperature, and lower values of K_m, thus showing greater affinity for *o*-nitrophenyl-β-D-galactopiranoside than the crude enzymes. The production of galactooligosaccharides was also greater when using purified enzymes, increasing the synthesis by more than 30 % by both strains.

Key words: inactivation kinetics, thermal properties, thermodynamic parameters

Introduction

β-Galactosidase (EC 3.2.1.23) is an enzyme that catalyzes the hydrolysis of lactose (abundant disaccharide found in milk) to glucose and galactose. It has potential importance due to various applications in the food dairy industries involving lactose-reduced ingredients (*1,2*). Major applications of β-galactosidase include improving the technological and sensory characteristics of foods by increasing their solubility, formation of galactooligosaccharides, assimilation of foods containing lactose for lactose-intolerant populations and the conversion of whey into different value-added products (*3*).

Galactooligosaccharides (GOS) are nondigestible oligosaccharides comprised of 2 to 20 molecules of galactose and one of glucose, which are recognized as prebiotics since they can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine (*4*). For this reason, much attention has been given to the production of GOS, especially *via* enzymatic transgalactosylation, since the chemical synthesis of GOS is very tedious (*5*).

β-Galactosidases are found in a variety of sources – animal, vegetable and microbial – and the enzyme characteristics vary according to their origin. The most technologically interesting β-galactosidases are produced

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by *Kluyveromyces* yeasts and are intracellular. Their synthesis is induced by galactose and repressed by glucose, and they are obtained mainly by submerged cultivation (*6,7*).

Good knowledge about the stability of an enzyme is an important aspect when considering its application in biotechnological processes, since it can provide information on the structure of the enzyme and facilitate an economical production design. Deactivation mechanisms can be complex, since enzymes have highly defined structures, and the slightest deviation from their native form can affect their specific activity. Better knowledge of enzyme stability under the operating conditions could help optimize the profitability of enzymatic processes (*8*).

The activity and thermal stability of enzymes are influenced by diverse environmental factors (temperature, pH, reaction medium, shaking) which can strongly affect the specific three-dimensional structure or spatial conformation of the protein (*8*). It is also important to analyze the estimated thermodynamic parameters, since this aids in understanding the probable denaturation mechanism, which is very important in enzymatic processes (*9*).

Several studies have already determined some of the properties and kinetic parameters of β-galactosidase, such as the deactivation rate constants (K_d), half-life ($t_{1/2}$), deactivation energy and the kinetic constants (K_m and v_{max}) (*3,6,8,9*). However, there are few papers in the literature considering the thermal and kinetic properties that compare crude and purified enzymes. Purification processes could modify enzyme properties in such way that their kinetic and thermodynamic behaviour could also be different. This could be associated with the removal of ligand and/or proteins that have a protective effect on the crude enzyme, and the removal of these components could decrease the thermostability or affinity for the substrate. On the other hand, the purification process could improve the specificity or synthetic capacity of the purified enzyme. Thus it is extremely important to acquire knowledge of the properties of both crude and purified enzymes in order to determine which one is the best when considering a specific use. Also, precipitation/dialysis and ion exchange chromatography used to purify β-galactosidase in the present study can and must be amenable for an industrial scale-up.

In this context, the properties of crude and purified β-galactosidase preparations obtained from two different sources, *Kluyveromyces marxianus* CCT 7082 and *Kluyveromyces marxianus* ATCC 16045, were analyzed. The strains had previously been selected as the best β-galactosidase producers amongst several others (*2,10*). The pH and temperature optima, deactivation energy, thermal stability and the kinetic and thermodynamic parameters were determined for both crude and purified enzyme solutions obtained from both strains, as well as their abilities to hydrolyze lactose and produce GOS.

Materials and Methods

Microorganism

Kluyveromyces marxianus CCT 7082, deposited in the Tropical Culture Collection of the Andre Tosello Foundation (Campinas, SP, Brazil), and *K. marxianus* ATCC 16045 were cultivated, and their enzymes were used in the studies below.

Inoculum

The cultures were grown on a medium containing (in g/L): lactose 10, KH_2PO_4 5, $(NH_4)_2SO_4$ 1.2, $MgSO_4 \cdot 7H_2O$ 0.4 and yeast extract 1 in 0.2 M potassium phosphate buffer, pH=5.5 (*11*). The medium was sterilized at 121 °C for 15 min and lactose was sterilized by filtration. A volume of 150 mL of inoculated medium was cultivated in conical flasks (500 mL capacity) for 14 h at 180 rpm and 30 °C in an orbital shaker (Tecnal TE-420, Piracicaba, SP, Brazil) (*12*).

Submerged cultivation

The enzyme was produced by submerged cultivation using the culture medium optimized by Manera *et al.* (*10*) containing (in g/L): lactose 28.2, KH_2PO_4 5.0, $(NH_4)_2SO_4$ 8.8, $MgSO_4 \cdot 7H_2O$ 0.4 and yeast extract 17.0 in 0.2 M potassium phosphate buffer, pH=6.0. Cultivations were started with 10 % of inoculum and the cultures were incubated for 96 h at 180 rpm and 30 °C in an orbital shaker (Tecnal TE-420).

Enzyme extraction

The enzymatic extract was distributed in 50-mL flasks containing 25 mL of cell suspension (40 mg/mL in 50 mM phosphate buffer plus 0.1 mM $MnCl_2 \cdot 4H_2O$, pH=6.6) (*13*) and 27.5 g of glass beads (r<0.4 mm), and mixed in a vortex (Fenix AP 56, Araraquara, SP, Brazil) (*14*) for 20 min with the temperature maintained at 4 °C. The suspension was then centrifuged at 5200×g for 10 min at 4 °C (Cientec CT-5000R, Porto Alegre, RS, Brazil), and the supernatant was assayed for β-galactosidase activity.

Enzyme purification

The enzymes from both strains were purified by precipitation, dialysis and ion exchange chromatography (IEC). The precipitation step was carried out as follows: ammonium sulphate was added to the solution containing β-galactosidase to 70 % saturation, maintained overnight and then centrifuged. The supernatant was discarded and the β-galactosidase-rich precipitate was dissolved in 0.05 M potassium phosphate buffer, pH=6.6, and dialyzed against the same buffer.

The extract containing the β-galactosidase obtained in the earlier step (after precipitation followed by dialysis) was loaded onto a column (C10/20, GE Healthcare Bio-Sciences, Uppsala, Sweden) packed with Q-Sepharose Fast Flow resin (GE Healthcare Bio-Sciences), previously equilibrated with 0.025 M potassium phosphate buffer, pH=7.5, with a flow rate of 20 cm/h. The unadsorbed proteins were removed by washing with 0.025 M potassium phosphate buffer (pH=6.5).

Elution was carried out in a stepwise mode, first using 18 mL of 0.1 M KCl to remove some of the protein contaminants, followed by a linear gradient 0.1–1.0 M KCl in 0.025 M potassium phosphate buffer (pH=6.5) at a volume of 95 mL. Samples (3 mL) were collected and fractions with higher activity pooled for subsequent char-

acterization. Following IEC, the pooled fractions were dialyzed against 0.05 M potassium phosphate buffer (pH=6.6). All experiments were carried out at 10 °C.

Effect of pH on β-galactosidase activity

The influence of pH on the enzymatic activity at 37 °C was determined by assaying the activity at different pH values ranging from 2.6 to 9.0, using 0.1 M concentrations of the following buffer systems: citrate phosphate (pH=2.6, 4.6 and 6.3), sodium acetate (pH=3.6, 4.6 and 5.6), sodium phosphate (pH=6.3, 7.3 and 8.0) and Tris-HCl (pH=7.3, 8.0 and 9.0). The relative activities (as percentage) were expressed as the ratio of the β-galactosidase activity obtained at a certain pH to the maximum activity obtained in the given pH range (*9*).

Effect of temperature on β-galactosidase activity

The effect of temperature on the activity of β-galactosidase was determined by carrying out the standard enzyme assay procedure at different temperatures ranging from 25 to 60 °C, the pH being fixed according to the buffer used in the analysis (0.1 M potassium phosphate buffer, pH=6.6). The substrate, *o*-nitrophenyl-β-D-galactopiranoside (ONPG, Sigma-Aldrich, St. Louis, MO, USA) was preincubated at the respective temperature for 5 min. The relative activities (as percentage) were expressed as the ratio of the β-galactosidase activity obtained at a certain temperature to the maximum activity obtained in the given temperature range.

Thermal deactivation kinetics and estimation of the deactivation energy

To investigate the thermal deactivation kinetics of β-galactosidase, crude and purified enzyme extracts were incubated at different temperatures (35, 37, 40, 45, 50 and 55 °C) in the absence of substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to the assay. The residual activity was expressed as a percentage of the initial activity.

From a semi-natural logarithmic plot of the residual activity *vs.* time, the deactivation rate constants (K_d) were calculated and the half-lives estimated using Eq. 1. The half-life ($t_{1/2}$) is defined as the time taken for the residual activity to reach 50 %.

$$t_{1/2} = \frac{-\ln 0.5}{K_d} \qquad /1/$$

The temperature dependence of K_d was analyzed using the Arrhenius plot and the deactivation energy calculated from the Arrhenius equation as:

$$\ln K_d = \ln A - \frac{E_d}{R}\left(\frac{1}{T}\right) \qquad /2/$$

where E_d is the deactivation energy of the transition state of enzyme deactivation, A is a constant and R the universal gas constant.

The value of the deactivation energy (E_d) was estimated from the slope of the plot of $\ln K_d$ against $1/T$.

Estimation of the thermodynamic parameters

The Gibbs free energy of inactivation (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*) of deactivation can be estimated by making use of the absolute reaction rates (*15*). The temperature dependency of the deactivation rate constant can be expressed as:

$$K_d = \frac{kT}{h}\exp\left(\frac{\Delta S^*}{R}\right)\exp\left(\frac{\Delta H^*}{RT}\right) \qquad /3/$$

$$\ln\left(\frac{K_d}{T}\right) = \ln\left(\frac{k}{h}\right) + \frac{\Delta S^*}{R} - \left(\frac{\Delta H^*}{R}\right)\frac{1}{T} \qquad /4/$$

where R is the universal gas constant, T the absolute temperature, h the Planck's constant ($6.6262 \cdot 10^{-34}$ J/s) and k the Boltzmann constant ($1.3806 \cdot 10^{-23}$ J/(mol·K)).

The values of ΔH^* and ΔS^* were calculated from the slope and intercept of the plot of $\ln(K_d/T)$ *vs.* $1/T$, respectively, and ΔG^* was estimated from the following relationship:

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad /5/$$

The values for the parameters D and z were also calculated to express enzyme deactivation. The D value can be calculated using the following equation:

$$D = \frac{2.3026}{K_d} \qquad /6/$$

The z value is the temperature interval of one logarithmic cycle on the denaturing curve and can be calculated from:

$$\log\frac{D_2}{D_1} = \frac{T_1 - T_2}{z} \qquad /7/$$

Determination of the kinetic constants

The kinetics of most reactions catalyzed by enzymes follows the Michaelis-Menten equation (*6*):

$$v = \frac{v_{max}[S]}{K_m + [S]} \qquad /8/$$

The K_m was determined using the double reciprocal ($1/V$ *vs.* $1/S$) Lineweaver-Burk plot with different substrate concentrations using ONPG as the substrate (1–10 mM):

$$\frac{1}{v} = \frac{K_m}{v_{max}}\frac{1}{S} + \frac{1}{v_{max}} \qquad /9/$$

β-Galactosidase assay

Intracellular β-galactosidase was obtained by an abrasive cell disruption technique using vortex with glass beads. β-Galactosidase activity was determined using ONPG as the substrate as described by Inchaurrondo *et al.* (*16*). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of *o*-nitrophenol (ONP) per min under the assay conditions. The liberated ONP was measured spectrophotometrically (Quimis Q-108D, Diadema, SP, Brazil) at 420 nm.

Lactose hydrolysis and galactooligosaccharide production

The crude and purified enzymes obtained from both sources were used for lactose hydrolysis and GOS production. Each enzyme (8 U/mL of solution) was added to the lactose solution (350 g/L of lactose in 0.1 M sodium phosphate buffer, pH=7.0). The flasks were incubated in a temperature-controlled shaking water bath at 40 °C for 24 h. Samples of the reaction mixture were removed at timed intervals and immediately immersed in a boiling water bath for 5 min to inactivate the enzyme before the sugar analysis. The synthesis medium was analyzed for its carbohydrate composition by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex (Thermo Scientific Dionex, Sunnyvale, CA, USA) chromatograph and a Carbopac PA-1 column equilibrated with 150 mM NaOH, eluted with 20 mM NaOH at a rate of 1 mL/min (*17*).

Results and Discussion

Purification results

Like many crude enzymatic extracts, β-galactosidase extracts contained a high concentration of proteins. The crude enzymes obtained from *Kluyveromyces marxianus* ATCC 16045 and CCT 7082 showed initial specific activities (U/mg) of 2.4 and 4.1, respectively. However, after the purification the specific activities (U/mg) of β-galactosidases were 19.1 and 19.3 for ATCC 16045 and CCT 7082 strains, respectively. The increase in the specific activities showed that proteins and other undesirable compounds had been removed from the enzymatic extracts obtained from both strains.

Influence of pH on β-galactosidase activity

The effect of pH on the activities of β-galactosidases obtained from *K. marxianus* CCT 7082 and ATCC 16045 was investigated for both the crude and purified extracts, as shown in Table 1. The optimum pH was determined to be 7.3 for all enzymes obtained from both sources and the buffer that showed excellent results for all the enzymes was 0.1 M sodium phosphate, although 0.1 M Tris-HCl buffer also showed high activity values for the crude enzyme from CCT 7082.

These findings are in agreement with earlier studies reported in the literature. In a study performed by Brady *et al*. (*18*), the optimum pH for the β-galactosidase from *Kluyveromyces marxianus* was found to be 7.5. Similar findings were observed in another study performed by Jurado *et al*. (*8*), where the optimum pH for the enzyme from *K. fragilis* was found to be 7.0.

It can be seen from the analyzed data that the purified enzymes were highly sensitive to pH changes. When the pH was not in the optimum range, both purified enzymes showed relatively large decrease in the activity, but this was not the case for the crude enzymes from both strains. The removal of proteases and protein fragments frequently present in the crude extract, which in some cases can be considered as enzyme-stabilizing substances, can promote the susceptibility of enzymatic activity to changes in the pH values. These results are in accordance with Lai *et al.* (*19*), who studied crude and purified superoxide dismutase which showed the same behaviour.

Table 1. Effect of pH on the activity of β-galactosidases from *Kluyveromyces marxianus* strains

Buffer (0.1 M)	pH	Enzyme relative activity/%			
		K. marxianus ATCC 16045		*K. marxianus* CCT 7082	
		crude	purified	crude	purified
acetate	2.6	11.1	0.1	0.2	0.1
	4.6	10.9	0.2	0.3	0.2
	6.3	79.6	3.0	98.1	1.5
citrate	3.6	10.7	0.1	0.5	0.1
	4.6	12.2	0.2	0.3	0.2
	5.6	23.2	5.5	96.0	6.0
phosphate	6.3	88.4	22.9	90.8	43.0
	7.3	100.0	100.0	95.9	100.0
	8.0	44.0	24.0	98.7	17.4
Tris-HCl	7.3	19.7	18.8	100.0	21.1
	8.0	17.2	15.5	99.4	14.3
	9.0	16.9	7.0	98.8	8.2

Influence of temperature on β-galactosidase activity

Comparing the strains, the crude enzyme from ATCC 16045 showed higher optimum temperature than that from CCT 7082, but when the purified enzymes were compared, both strains showed the same range of optimum temperature (Fig. 1).

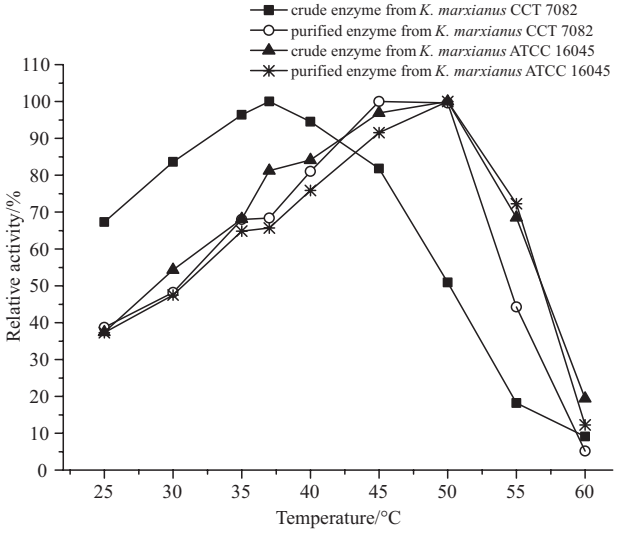

Fig. 1. Effect of temperature on the activity of crude and purified β-galactosidases

Crude β-galactosidases from CCT 7082 and ATCC 16045 were found to have optimum temperatures of 37 and 50 °C, respectively. The optimum temperatures of purified β-galactosidases from CCT 7082 and ATCC

16045 were 45 and 50 °C, respectively. The CCT 7082 strain showed a huge increment in optimum temperature after purification, indicating a tendency to be more thermostable.

Similar findings have been reported in several studies, Brady *et al.* (*18*) found the optimum temperature for β-galactosidase from *K. marxianus* to be between 40 and 50 °C, showing similar behaviour to the optimum temperature found for the crude and purified enzyme extracts from ATCC 16045 and the purified enzyme extract from CCT 7082. In addition, some other studies found the optimum temperature for enzyme extract to be similar to that found in the present study for crude enzyme extract from CCT 7082, such as Jurado *et al.* (*8*), who found a value between 25–40 °C for β-galactosidase from *Kluyveromyces fragilis*.

Kinetics of thermal deactivation and estimation of the deactivation energy

Deactivation is known to be a process where the secondary, tertiary or quaternary structure of a protein changes without breaking any covalent bonds. The deactivation rate constants (K_d) of the crude and purified β-galactosidases from CCT 7082 and ATCC 16045, shown in Table 2 for the temperatures of 30, 35, 37, 40, 45, 50 and 55 °C, were calculated from the slope of the semi-natural logarithmic plot of residual activity *vs.* time. Similarly, the half-life ($t_{1/2}$) values were calculated using the deactivation rate constants, also presented in Table 2.

The half-lives obtained for the purified extract from ATCC 16045 strain increased at almost all the temperatures when compared to the crude extract. Considering the optimum temperature, the half-lives increased more than 2-fold. The CCT 7082 strain showed the same behaviour, but the increase was more significant since the half-lives increased by more than 20-fold in the optimum temperature range.

A comparison of the half-lives of the crude and purified enzymes at different temperatures showed that the enzyme from *K. marxianus* ATCC 16045 was more stable than that from *K. marxianus* CCT 7082. However, considering just the crude enzymes, that obtained from CCT 7082 showed greater stability at its optimum temperature (37 °C) than that obtained from ATCC 16045 (50 °C).

Alcántara *et al.* (*20*), studying lipases (crude and partially purified), obtained similar results to the present ones. The partially purified *Rhizomucor miehei* lipase showed enhanced thermostability when compared to the crude enzyme (8.7- and 1.8-fold, considering the half-life times at 37 and 50 °C, respectively). Lai *et al.* (*19*) observed that the purified enzyme was slightly more thermally stable than crude superoxide dismutase, in accordance with the data shown in the present study.

On the other hand, Naidu and Panda (*21*) showed that crude pectolytic enzymes were more stable than the partially purified enzymes. According to these authors, this could be due to: (*i*) interaction effects amongst the enzyme components; (*ii*) other proteins besides those components secreted by the organism; and/or (*iii*) a combination of the two. This contradiction between the present data and those of other authors showed the importance of studying the properties of both crude and purified enzymes, since depending on the enzyme and its application, purification could be appropriate or not.

The deactivation energy (E_d) of crude β-galactosidases from CCT 7082 and ATCC 16045 was found to be 422.5 and 327.7 kJ/mol, respectively, and that of the purified enzymes was in approximately the same range. The E_d value of the purified enzyme from ATCC 16045 was 400.8 kJ/mol and of that from CCT 7082, it was 351.3 kJ/mol. These values are in the same range (200–600 kJ/mol) estimated for many enzymes (*15*).

Thermodynamic parameters

It is essential to study β-galactosidases from different strains in order to determine the thermodynamics and thermostability properties, and hence apply the β-galactosidases obtained from microbial sources that show the most promising industrial potentials. It is also important to understand the structure-stability relationships of their enzymes. An estimation of the thermodynamic parameters helps to understand the probable denaturation mechanism, which is very important in enzymatic processes (*22*). Table 3 shows the changes in enthalpy (ΔH^*) and entropy (ΔS^*) occurring during the thermal inactivation of crude and purified β-galactosidase, calculated using the transition state theory (*21*).

Table 2. Kinetic parameters for the thermal deactivation of crude and purified β-galactosidases from *Kluyveromyces marxianus* strains

Temperature / °C	K_d/min^{-1}				$t_{1/2}$/h			
	K. marxianus ATCC 16045		*K. marxianus* CCT 7082		*K. marxianus* ATCC 16045		*K. marxianus* CCT 7082	
	crude	purified	crude	purified	crude	purified	crude	purified
30	–	–	0.0002	0.0001	–	–	57.7	115.5
35	0.0003	0.0001	0.0008	0.0001	38.5	115.5	10.5	115.5
37	0.0003	0.0001	0.0048	0.0003	38.5	115.5	2.1	38.5
40	0.0004	0.0002	0.0290	0.0006	28.9	57.8	0.9	19.3
45	0.0253	0.0014	0.3992	0.0061	0.46	8.3	0.08	1.9
50	0.0830	0.0480	–	0.0861	0.14	0.24	–	0.13
52	–	0.2746	–	0.1159	–	0.04	–	0.10
55	0.3129	0.3815	–	0.4176	0.04	0.03	–	0.03
60	–	0.9886	–	1.2011	–	0.01	–	0.01

Table 3. Values obtained for the thermodynamic and kinetic parameters of the thermal deactivation of crude and purified β-galactosidases from *Kluyveromyces marxianus* strains at 30–55 °C

Enzyme	ΔG^*	ΔH^*	ΔS^*	D	K_m[#]
	kJ/mol	kJ/mol	kJ/(mol·K)	min	mM
ATCC 16045 crude	106.8–94.9	325.0	0.712	7.4–7666.7	3.3
ATCC 16045 purified	109.7–94.3	398.2	–	6.1–23000	2.3
CCT 7082 crude	109.7–91.3	419.9	1.030	8.7–11500	9.9
CCT 7082 purified	109.7–94.1	354.0	0.793	5.5–7666.7	3.3

[#]ONPG in phosphate buffer (50 mM with 0.1 mM $MnCl_2 \cdot 4H_2O$, pH=6.6)

Assuming a hydrogen or hydrophobic bond, the removal of a $-CH_2$ group by solvent contact at a strength of about 5.4 kJ/mol (*23*) and the formation of transition state in the β-galactosidases from ATCC 16045 and CCT 7082 would be accompanied by the disruption of up to 60 and 58 non-covalent bonds, respectively.

As can be seen in Table 3, with an increase in temperature, a slight decrease in the values of ΔG^* occurred for both strains in the crude and purified forms. All the ΔG^* values were to the expected order of magnitude for protein denaturation (*24*). Ustok *et al.* (*9*) reported similar findings, with ΔG^* values of 99.7–105 kJ/mol for the β-galactosidases produced by artisanal yoghurt cultures.

Solvent and structural effects are the two major factors influencing the numerical values of ΔH^* and ΔS^* (*22*). Table 3 shows that the values of ΔH^* and ΔS^* obtained for both strains in the present study were in general agreement with the values expected for enzyme heat-inactivation. The large activation enthalpy values were also characteristic for protein denaturation (*25*) and the ΔS^* values were close to zero, showing that thermal deactivation did not imply any relevant variation in the tertiary structure of the enzyme (*9*).

When working with enzymes employed in the food industry, their deactivation is commonly expressed in terms of the values for the parameters D and z (*26*). The D or decimal reduction value (in min) is defined as the time the enzyme must be preincubated at a given temperature to maintain 10 % residual activity, and was calculated using Eq. 6. Since β-galactosidase is used in dairy industries for products whose preparation sometimes requires high temperatures, it was considered convenient to determine the D values, as shown in Table 3. The values calculated for z (the temperature interval required for the thermal inactivation curve to pass through one log cycle) were 6.41 and 5.7, 6.73 and 6.42 °C for CCT 7082 (crude and purified) and ATCC 16045 (crude and purified), respectively. The z value was lower for the purified enzymes, showing that these are more sensitive to temperature changes.

Kinetic constant determination

The kinetic parameters of the crude and purified β-galactosidases from CCT 7082 and ATCC 16045 in the hydrolysis of ONPG were obtained using a typical double reciprocal Lineweaver-Burk plot (*27*). The K_m values were calculated and shown in Table 3. The value estimated for the K_m of the crude enzyme from CCT 7082 was five times higher (lower affinity) than that of the enzyme from ATCC 16045. This indicates that the crude β-galactosidase from ATCC 16045 had a greater affinity for ONPG than that from CCT 7082. The values of K_m of the crude enzyme from ATCC 16045 were to the same order of magnitude as the values of β-galactosidases from other microbial sources. Zhou and Chen (*28*) and Nagy *et al.* (*29*) obtained values for the K_m of the galactosidases from *K. lactis* and *Penicilium chrysogenum* of 1.7 and 1.8 mM, respectively, using the substrate ONPG.

The K_m value of the purified enzyme from ATCC 16045 was slightly lower than that obtained for the crude enzyme, showing similar behaviour considering its affinity for the substrate (ONPG). However, in the case of the enzyme from CCT 7082, the K_m values observed for the purified enzyme were much lower than those obtained for the crude one, indicating greater affinity for ONPG. This behaviour could be related to the removal of contaminant proteins present in the enzymatic extract, which could be competing with the target enzyme.

Determination of lactose hydrolysis and galactooligosaccharide production

The enzymes, crude and purified, and the lactose solution were incubated together to determine the degree of hydrolysis of the lactose and the production of GOS (Fig. 2).

It was observed that under the tested conditions both crude enzymes hydrolyzed lactose into glucose and galactose and produced GOS. The hydrolysis of lactose increased up to 10 h of reaction time, at which point approx. 90 % of the lactose had been converted. The greatest production of GOS was between 3 and 4 h, obtaining values of 40 and 54 g/L for the crude enzymes from *K. marxianus* ATCC 16045 and CCT 7082, respectively. After this reaction time, there was a decrease in the GOS concentration due to hydrolysis.

The purified enzymes showed similar behaviour with respect to the hydrolysis of lactose into glucose and galactose and the production of GOS. However, the greatest production of GOS was between 4 and 6 h, obtaining better production than that from the crude enzymes, reaching values of 55 and 72 g/L for the purified enzymes from *K. marxianus* ATCC 16045 and CCT 7082, respectively, values 37.5 and 33.3 % higher, respectively, than the values obtained for GOS from the crude extracts. Both enzymes from both strains showed maximum activity at neutral pH, suggesting that the enzymes were

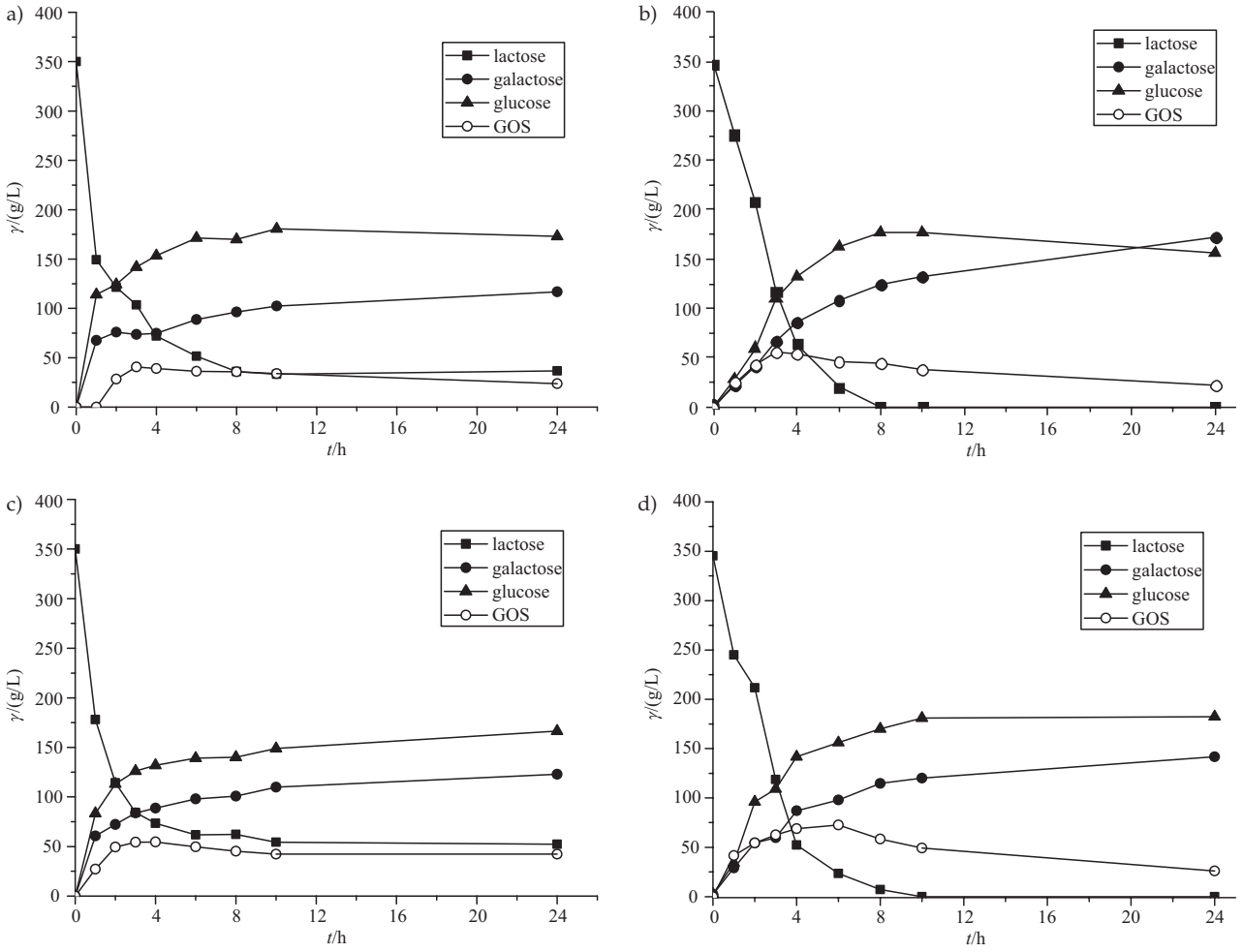

Fig. 2. Lactose hydrolysis and GOS production by: a) the crude enzyme from *Kluyveromyces marxianus* ATCC 16045, b) the purified enzyme from *K. marxianus* ATCC 16045, c) the crude enzyme from *K. marxianus* CCT 7082, and d) the purified enzyme from *K. marxianus* CCT 7082

ideal candidates for the hydrolysis of lactose in milk, and would be suitable for application in low-lactose milk production enriched with GOS.

Conclusions

The properties of two β-galactosidase enzymes, crude and purified, obtained from different sources, *K. marxianus* CCT 7082 and *K. marxianus* ATCC 16045, were determined. The results showed the importance of purification process, as it affects the activities of these enzymes. The purified enzymes showed higher optimum temperatures. Considering the kinetic properties, the purified enzymes showed lower values of K_m, hence they showed greater affinity for ONPG than the crude enzymes. The production of GOS was also greater when using purified enzymes. As can be seen, there were some disadvantages in the purification process as shown by the decrease in the activity when the pH value and temperature were outside the optimum ranges. In addition, lower *z* values obtained with the purified enzymes in relation to those obtained with the crude enzymes showed a more sensitive behaviour when the temperature changes, confirming the importance of determining the characteristics and parameters of both crude and purified enzymes when dealing with enzymes such as β-galactosidase for use in biotechnological processes.

Acknowledgements

The present work was carried out with the financial support of CAPES, CNPq and FAPERGS, all Brazilian government entities.

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