

Thermal inactivation kinetics of *Aspergillus oryzae* β -galactosidase in concentrated lactose solution

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Abstract: Thermal inactivation of a commercial β -galactosidase from *Aspergillus oryzae* in a 300 g/L lactose solution was studied in the temperature range of 65–75 °C. Lactose exhibited a stabilisation effect when similar inactivation rates as those in lactose solution were observed in a lactose-free solution at temperatures lower by 5 °C. Inactivation process in the lactose solution was biphasic. A kinetic model based on the Lumry-Eyring mechanism was proposed and successfully verified. Estimated activation energy values were very different. Rather high activation energy values of the forward reactions were responsible for both the significant change of rate constants and the rate-controlling reaction with temperature. For these two reasons, an increase of the operational lifetime of the enzyme from 7 days at 60 °C to 580 days at 55 °C was predicted.

Keywords: β -galactosidase, thermal inactivation, kinetics, Lumry-Eyring mechanism, multitemperature modelling

Introduction

β -Galactosidase (β -GAL) is an enzyme with several important industrial applications. One of them is the production of lactose-free dairy products for people with lactose intolerance. Other applications that exploit the hydrolytic activity of β -GAL toward lactose include industrial processing of whey for ethanol production or dairy wastewater processing. Finally, prebiotic compounds, galactooligosaccharides (GOS), are also produced from concentrated lactose solutions using the transgalactosylation activity of β -GAL.

Elevated temperature is beneficial in all these enzymatic processes since it results in increased reaction rate and bioreactor productivity. It offers additional advantages for the production of GOS, increases lactose solubility and decreases the viscosity of reaction medium. Since β -GAL, as each enzyme is a thermolabile compound, the reaction temperature has to be strictly optimised to find a compromise between good activity and stability.

Thermal stability of β -GAL in its free or immobilised form has been investigated by a number of authors. Its stability varies significantly among different microbial producers of the enzyme. Flaschel (2006) studied the stability of three different β -GAL forms during UHT milk lactose hydrolysis. He found that *K. lactis* β -GAL is the most thermolabile form which is sufficiently stable only below 40 °C. Enzymes from *A. oryzae* and *E. coli* are stable up to 50 °C. Osman et al. (2014) studied the stability of *E. coli* β -GAL during the production of GOS and

found that a complete conversion can be achieved at the temperature of 55 °C.

K. marxianus β -GAL preparations have sufficient stability in a phosphate buffer solution only at temperatures below 40 °C (Braga et al., 2013).

A similar observation was made by Ladero et al. (2006) for a commercial *K. fragilis* β -GAL. The stability of purified preparations was however several times higher than that of their crude counterparts. *B. circulans* β -GAL has a half-life of only about 5 min at 60 °C which increased to 4 h for an immobilised form of this enzyme (Urrutia et al., 2013).

Nakanishi et al. (1983) presented the half-lives of 50 days at 50 °C and 15 days at 55 °C for an immobilised form of *B. circulans* β -GAL. Specific results were achieved for highly thermostable β -GAL from *Thermus sp.* which has a half-life of about 1 h at 90 °C and can withstand temperatures close to the solution boiling point for a short period of time (Ladero et al., 2005; Ladero et al., 2006).

The objective of this work was to investigate the inactivation of a commercial β -GAL from *A. oryzae* in a concentrated lactose solution at different temperatures and to determine a suitable inactivation mechanism. The resulting time-activity data were processed using the so-called multitemperature evaluation method (Vrábel et al., 1997; Polakovič and Bryjak, 2002), which can be successfully described by a complex inactivation model (Illeová et al., 2003).

Inactivation experiments were carried out also in a lactose-free solution to determine the stabilisation effect of this saccharide.

Materials and methods

Materials

β -GAL powder Tegaferm LAC A100P from *A. oryzae* with estimated initial activity of 100 000 U g⁻¹ was purchased from Tegaferm Holding GmbH (Wien, Austria). Lactose monohydrate was obtained from Centralchem (Bratislava, Slovakia), *o*-nitrophenyl- β -D-galactopyranoside (ONPG) and 2-nitrophenol were provided by Sigma-Aldrich (St. Louis, USA). All other reagents were of analytical grade and were obtained from Mikrochem (Pezinok, Slovakia). Acetate buffer was prepared from 0.1 M acetic acid and adjusted to pH 4.5 using 0.1 M sodium acetate. Enzyme stock solution in the acetate buffer was prepared at the β -GAL concentration of 3 g/L.

Inactivation experiments

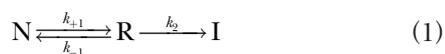
Enzyme inactivation experiments were carried out either in a reaction medium with the initial lactose concentration of 300 g/L or in the lactose-free acetate buffer. The temperature range used was 65–75 °C. About 15 mL of the liquid was preheated to the inactivation temperature and 1 mL of the enzyme stock solution was added. Sampling frequency was adjusted to the dynamics of the inactivation process. The sample volume was 0.7 mL. Enzyme inactivation was stopped by cooling the samples in ice water.

Determination of enzyme activity

Residual activity of enzyme samples was measured at 37 °C using the initial rate method when a 3.7 g/L ONPG solution in the acetate buffer was used as the substrate. The enzymatic reaction was stopped by adding 10 % sodium carbonate. The concentration of 2-nitrophenol released was determined using a UV-Vis spectrophotometer at the wavelength of 420 nm. One unit of β -GAL activity corresponded to 1 micromole of 2-nitrophenol produced from ONPG by β -GAL per minute.

Mathematical modelling

In this study, a kinetic model (Onderková et al., 2010) was examined for the description of the courses of relative enzyme activity, a , defined as the ratio of c_N , the concentration of native enzyme form, N, in time, t , and c_{N0} , its initial concentration. This model was based on the mechanism:



where N undergoes a reversible transformation into the intermediate inactive form, R, which then reacts irreversibly to the inactive form, I. Symbols k_{+1} , k_{-1} , and k_2 are the rate constants of individual reactions. The mechanism has the same form as the

well-known mechanism of Lumry-Eyring (1954) but the interpretation is different. In the Lumry-Eyring mechanism, the intermediate is considered to be a fully unfolded enzyme and rapid equilibrium is typically assumed for the first reaction. The inactivation process was described by a set of the following differential equations:

$$\frac{dc_N}{dt} = -k_{+1}c_N + k_{-1}c_R \quad (2a)$$

$$\frac{dc_R}{dt} = k_{+1}c_N - k_{-1}c_R - k_2c_R \quad (2b)$$

$$t = 0, c_N = c_{N0}, c_R = 0 \quad (2c)$$

Temperature dependence of the rate constants was expressed in form of a rearranged Arrhenius equation,

$$k_i = e^{\ln k_{i0}} e^{[(E_i/RT_0)(1-T_0/T)]}, i = +1, -1, 2 \quad (3)$$

where E_i is the activation energy of the i th reaction, R is the universal gas constant, and k_{i0} the value of rate constant k_i at reference temperature $T_0 = 343.15$ K. The so-called multitemperature evaluation (Vrábel et al., 1997; Polakovič and Bryjak, 2002), where inactivation data for all temperatures are fitted simultaneously using non-linear regression, was applied. The mathematical modelling software Athena Visual Workbench (Stewart & Associates, Madison, WI, U.S.A.) was used for parameter estimation employing a gradient non-linear least square method and parameter uncertainties calculation based on the variance-covariance matrix.

Results and discussion

Fig. 1 displays the results of batch inactivation experiments of β -GAL in concentrated lactose solution in the temperature range of 65–75 °C. Fast exponential decline of activity was observed at higher temperatures when the half-life was only 0.5 min at 75 °C and 4 min at 70 °C. At two lower temperature values, biphasic character of inactivation was observed which was strongly pronounced at 65 °C. The half-life at 65 °C was 75 min but the enzyme still kept 25 % of its initial activity after 8 hours. These values represent very good thermal stability compared to that of other common β -galactosidases presented in Introduction. Flaschel (2006) determined the half-life of *A. oryzae* β -GAL from another source to be 1 h at 60 °C but all activity was lost after 4 h since the inactivation process was governed by first-order kinetics.

In order to evaluate the stabilisation effect of lactose, inactivation was carried out also in a lactose-free buffer (Fig. 2), where the inactivation was evidently faster. The stabilisation effect of the 300 g/L lactose

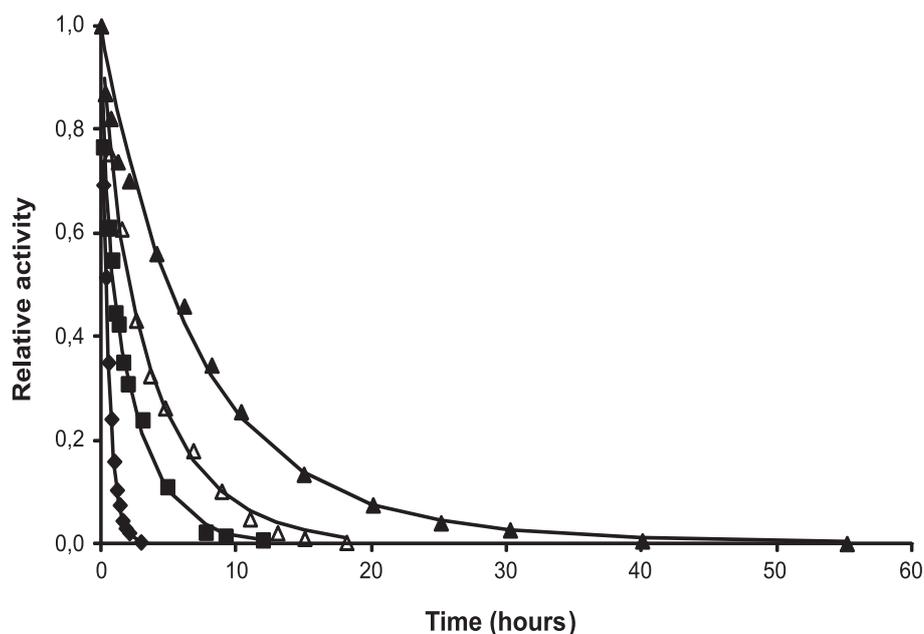


Fig. 1. Thermal inactivation of β -GAL in a reaction medium with the initial lactose concentration of 300 g/L. Symbols represent experimental data at individual temperatures:
 ● – 75 °C, ■ – 70 °C, Δ – 67.5 °C, \blacktriangle – 65 °C.
 Lines are the fitted activity values using the model specified by Eqs. (1)–(3).

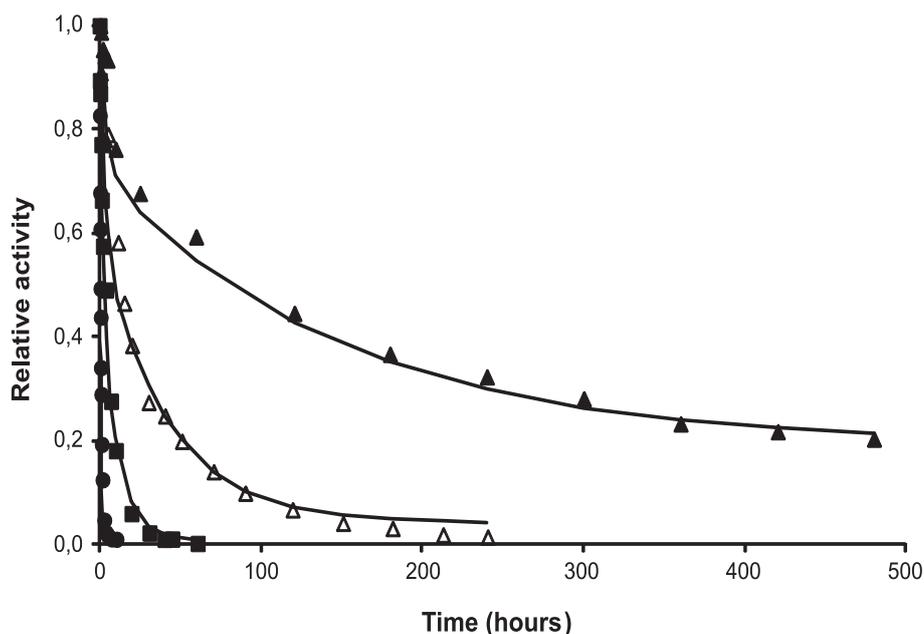


Fig. 2. Thermal inactivation of β -GAL in lactose-free buffer. The meaning of all symbols and lines is the same as in Fig. 1 except for the experimental data at 72.5 °C (\blacklozenge).

solution can be estimated to be about 5 °C. This degree of stabilisation is in compliance with the trend of saccharide concentration on enzyme inactivation in our previous study (Madlová et al., 2000).

A stabilisation effect of about 2 °C was observed for the inactivation of pectin methylesterase in different fruit juices containing about 10–15 % of saccharides (Wilińska et al., 2008).

A much stronger stabilisation effect was observed

for fructosyltransferase inactivation in a 700 g/L saccharide solution (Onderková et al., 2010).

It was estimated to be from 8 °C at higher temperatures to 15 °C at lower temperatures.

Experimental inactivation data were fitted with the model described above in the Mathematical modelling section. Estimated parameter values for the inactivation in the concentrated lactose solution and their 95 % confidence intervals are pre-

sented in Table 1. Mean square error of the activity values was 2.6 %. Fig. 1 demonstrates a good match between the measured and fitted activity values. Unfortunately, activation energy of the backward reaction, E_{-1} , could not be estimated reliably. It had to be fixed to get good statistical characteristics of the remaining model parameters. This is a common problem for models based on the Lumry-Eyring mechanism (Polakovič and Bryjak, 2002; Onderková et al., 2010).

The estimated activation energies E_1 and E_2 can be compared with the values presented by other authors. Ladero et al. (2006) dealt with the investigation of *K. fragilis* β -GAL in different solutions. Typical values of activation energy were from 250 kJ mol⁻¹ to 400 kJ mol⁻¹. Braga et al. (2013) give values from 330 kJ mol⁻¹ to 420 kJ mol⁻¹ for several β -GAL preparations from *K. marxianus*. A broad range of activation energy values, from 270 kJ mol⁻¹ to 940 kJ mol⁻¹, were reported by Flaschel (2006) for β -GAL from three different microbial sources. The value given for *A. oryzae* β -GAL, 515 kJ mol⁻¹, is essentially the same as that of E_2 in this work (Table 1).

Tab. 1. Model parameters of β -GAL inactivation kinetics in a reaction medium with the initial lactose concentration of 300 g/L.

Parameter	Value \pm 95 % confidence interval
k_{+10} (min ⁻¹)	0.305 \pm 0.034
k_{-10} (min ⁻¹)	0.127 \pm 0.044
k_{20} (min ⁻¹)	0.132 \pm 0.062
E_{-1} (kJ mol ⁻¹)	307 \pm 27
E_1 (kJ mol ⁻¹)	23 \pm n.d.
E_2 (kJ mol ⁻¹)	518 \pm 93

n.d. - not determined.

Since the value of E_{-1} is substantially lower than that of E_2 , temperature will play a significant role in the inactivation rate-controlling steps. Table 1 shows that the reaction rate constants at the reference temperature of 70 °C are not very different. The equilibrium constant of the first reaction, K_1 , at this temperature is equal to 2.4. The reversible character of the first reaction was thus manifested at this temperature and the dynamics of activity loss was influenced by both steps of the series reaction. Due to the low activation energy of the backward reaction, E_{-1} , the dependence of K_1 on temperature was determined primarily by the value of E_{+1} . The equilibrium constant thus increases rather quickly with the temperature and the equilibrium of the first reaction is strongly shifted toward the formation of the inactive inter-

mediate R. K_1 reached the value of 10 at 75 °C and the first reaction became apparently irreversible. Thus, the first-step is the rate-controlling step at high temperatures.

An opposite trend can be observed when the inactivation process is carried out at lower temperatures. As it has been mentioned above, the activity course at 65 °C had a bend at the residual activity of about 70 %. It can be assumed that the first step approached an apparent equilibrium, which correlates very well with the value of $K_1 = 0.55$. The rate constant k_{+1} was about seven times higher than k_2 . The slower rate of the second phase of inactivation was thus determined primarily by the rate of the second step. To be more precise, the activity course in the second phase can be approximated by the following equation (Gibbs et al., 2005):

$$a = \frac{e^{-\frac{K_1 k_2 t}{K_1 + 1}}}{K_1 + 1} \quad (4)$$

Experimental lifetime of several hours observed at 65 °C appears to be acceptable for the use of this enzyme preparation in its soluble form for the production of GOS (Braga et al., 2013; Osman et al., 2014).

It is however worth to emphasize that the significant inactivation during the production process decreases its productivity and total turnover number of the biocatalyst. The effect of temperature on the rate of the GOS synthesis is much weaker compared to that on the inactivation rate. A typical value of activation energy for GOS synthesis by free β -GAL is 20 kJ mol⁻¹ (Osman et al., 2014).

A change of temperature by 5 °C thus results only in a 10 % change of the GOS synthesis rate. For these reasons, the temperature of 65 °C may not be the optimal one.

Lower temperatures are necessary if this enzyme was applied in the immobilised form since the immobilisation costs have to be compensated by the repeated or continuous use of the biocatalyst (Urrutia et al., 2013).

Economic benefits of the immobilised β -GAL operational lifetime of one year or longer were presented a long time ago (Yang and Okos, 1989).

Although many advanced immobilisation methods increase the thermostability of β -GAL (Urrutia et al., 2013), many common methods based on commercial carriers did not change the enzyme's stability (Giacomini et al., 1998; Adamíková et al., 2018). It is therefore meaningful to use the model obtained for the simulation of β -GAL performance at temperatures below 65 °C.

High activation energy values of the forward reactions, E_{+1} and E_2 , underscore the fact that the

β -GAL inactivation rate should decrease rather strongly at lower temperatures. This effect is even more enhanced by the low activation energy of E_1 as it was discussed above. The calculated value of K_1 was only 0.12 at 60 °C and 0.005 at 50 °C. The first phase becomes then absent in the inactivation course and Eq. (4) is simplified to

$$a = e^{-K_1 k_2 t} \quad (5)$$

Thus obtained inactivation curve has the form of a simple exponential relationship but it cannot be considered as first-order kinetics since the exponential parameter is the product of the rate constant k_2 and equilibrium constant K_1 . The inactivation process is controlled by the rate of the second reaction but this proceeds very slowly because the concentration of intermediate R is very low.

Eq. (5) can then be used to calculate the β -GAL half-life $t_{1/2}$ at lower temperatures:

$$t_{1/2} = \frac{\ln 2}{K_1 k_2} \quad (6)$$

As expected, the predicted half-life increases very quickly in the direction toward lower temperatures. At 60 °C, $t_{1/2}$ is 7 days which implies that the activity loss of free enzyme in a batch process lasting several hours has a small effect on the bioreactor productivity and enzyme consumption. The predicted half-life of 580 days at 55 °C meets the requirement for immobilised β -GAL. Of course, uncertainty of these predictions is influenced by the problem with the reliability of the estimated value of E_1 . Nonetheless, the temperature of 55 °C is a good first estimate for experimental verification of operational temperature of immobilised β -GAL preparations screened in our recent study (Adamíková et al., 2018).

Conclusions

This work deals with thermal inactivation of a commercial β -GAL from *A. oryzae* in a concentrated lactose solution in the temperature range of 65–75 °C. Inactivation experiments in lactose-free buffer showed that a 300 g/L lactose solution has a stabilisation effect on β -GAL. Experimental activity courses were used to suggest a plausible mechanism of inactivation and identify kinetics of the inactivation process in the lactose solution. Since the inactivation course was clearly biphasic at the temperature of 65 °C, a model based on the two-step Lumry-Eyring mechanism was specified. Using the multitemperature method of inactivation data evaluation, a good fit was obtained for this model.

The verified model provided the pre-exponential rate constant and activation energy values to be used to determine the influence of individual rate

constants of the two-step reaction mechanism at different temperatures. It can be inferred from them that the first reaction becomes essentially irreversible at temperatures above 75 °C. The inactivation rate is then governed by the rate constant of the first reaction. On the contrary, the model results extrapolated into the region of lower temperatures imply that the inactivation is governed by the second, irreversible, reaction at temperatures below 60 °C. The predicted enzyme half-life increases quickly from the experimental value of 75 minutes at 65 °C to 7 days at 60 °C and 580 days at 55 °C. These predictions have been the starting point in our further study on the operational stability of immobilised β -GAL which is currently under progress.

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