ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 19–20 (2002) 443–450

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Modelling of the kinetics of thermal inactivation of glucoamylase from *Aspergillus niger*

Milan Polakovič [a,*], Jolanta Bryjak [b]

[a] *Department of Chemical and Biochemical Engineering, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic*

[b] *Institute of Chemical Engineering, Technical University of Wroclaw, ul. Norwida 4/6, 50-373 Wroclaw, Poland*

Received 28 September 2001; received in revised form 24 May 2002; accepted 7 June 2002

Abstract

Thermal inactivation of glucoamylase (GA) from *Aspergillus niger* was investigated by incubating the enzyme in buffer solutions in a broad temperature range. Preliminary experiments, made at different initial enzyme concentrations, showed the absence of association/aggregation reaction in the inactivation pathway. A biphasic character of inactivation was identified owing to the frequent sampling, conduction of experiments until the almost complete activity loss, and good accuracy of activity measurement. A further kinetic analysis showed that the biphasic inactivation could not be explained be the behaviour of different isozyme forms. The method of multitemperature evaluation was then applied to fit simultaneously all data with a model based on the Lumry–Eyring mechanism that incorporated the influence of temperature on the rate constants through the Arrhenius equation. A very close fit of activity values and good accuracies of the model parameters, the rate constants at the reference temperature of 60 °C and activation energies, validated the use of the model for the description of thermal inactivation of glucoamylase inactivation.

Keywords: Enzyme inactivation; Glucoamylase; Lumry–Eyring mechanism; Kinetics; Mathematical modelling

1. Introduction

Glucoamylase (GA; 1,4-α-D-glucan glucohydrolase EC 3.2.1.3) is an important enzyme in starch processing; used in a soluble form at elevated temperatures and prolonged times when parallel hydrolytic and inactivation reactions take place [1]. Many studies have been devoted to one of the most common glucoamylases from *Aspergillus niger*. It is well established nowadays that the natural preparations of this enzyme consist of two monomeric, glycosylated isozyme forms (GA1 and GA2, respectively) having different length and structure of amino acid residues [2]. Both isozyme forms aggregate at pH below 5 [2]. Sasvári et al. [3] found that GA2 formed active dimers during thermal inactivation at pH 4.5. Kinetic studies presented so far the activity loss to be a first-order one-step irreversible process [3–7]. Várallyay et al. [5] showed that the differences between the rate constants of GA1 and GA2 inactivation were minimal. Christensen et al. [2] who investigated the unfolding of isolated forms by differential scanning calorimetry, found that at pH 6.0, GA1 unfolded in a one-step irreversible process whereas GA2 exhibited a two-step irreversible transition. A slightly biphasic pattern of inactivation with a short first phase was presented

* Corresponding author. Tel.: +421-2-59325254; fax: +421-2-52496743.
E-mail address: polakovic@chtf.stuba.sk (M. Polakovič).

1381-1177/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1381-1177(02)00197-2

by Szajáni et al. [8] but they did not evaluate the kinetics.

It has been demonstrated in previous publications of the first author that a bad design of inactivation experiments and/or their inconvenient evaluation cause a failure in identifying more complex inactivation mechanisms [9–11]. The terms isothermal and multitemperature evaluation, respectively, were coined in these studies and will be used also here to stress the shortcomings of the conventional evaluation of a single inactivation curve at one temperature as compared to the simultaneous evaluation of inactivation curves obtained at different temperatures. Some other authors utilized the principle of multitemperature evaluation without using this term [12–16]. Schokker and Vanboekel [13] used it in a same manner as in our above mentioned studies. Others used this principle in the kinetic studies of more complex systems where other phenomena, typically parallel enzyme reactions, accompanied enzyme inactivation [12,14,16]. An interesting modification of the principle of probing and evaluating of inactivation kinetics in a broader temperature range was presented by Boy et al. [15] who applied a programmed temperature change in a bioreactor with controlled substrate concentration.

The objective of this study was to identify a possible mechanism and evaluate the kinetics of inactivation of soluble GA in a buffer solution that could be used for further studies of the kinetics of starch hydrolysis affected by enzyme inactivation. In order to achieve this goal, a number of inactivation batch experiments at different temperatures and initial enzyme concentrations were performed which were then processed simultaneously.

2. Experimental

2.1. Materials

Aspergillus niger glucoamylase of technical grade (Amigase, series 8187/SPE 0551) with specific activity of 0.443 U/mg of protein was a gift from Gist-Brocades (Seclin Cedex, France). The technical preparation was diluted 2500 times in a 0.05 mol dm^{-3} acetate buffer as a stock solution. Soluble starch was from Aldrich (Steinheim, Germany); sodium acetate, acetic acid and glucose assay kit (Glukoza EO, glucose oxidase–peroxidase method) were purchased from POCh (Gliwice, Poland).

2.2. Methods

Kinetic experiments were performed in a thermostated laboratory reactor equipped with a stirrer at different temperatures. A specific volume of the enzyme stock solution was added into 50 cm^3 of preheated acetate buffer of pH 5.2 in order to achieve the initial GA protein concentration in the reactor from 0.12 to 4 mg dm^{-3}. A vigorous stirring of the batch content was applied during the first 10 s and then stopped. In certain time intervals, aliquots were taken, cooled rapidly in ice water bath and stored in it (for about 1–1.5 h) until the activity measurement. It has been checked that no change of activity occurred during this storage time.

GA activity in assayed samples was determined by the initial reaction rate method at the temperature of 25 °C in the 1.25 mass% gelatinised soluble starch solution. The reaction time was set to match the expected activity (from 5 min to 12 h). The concentration of released glucose was measured by the glucose oxidase–peroxidase method. One unit of activity (U) was defined through the molar amount of released glucose at the conditions given above. The protein concentration of the enzyme was determined by Lowry's method [17].

Since the first assay was taken in the time of 10 s, the initial activity was an extrapolated value obtained by smoothing the initial part of the time versus activity dependence with an exponential relationship. Mathematical modelling software Athena Visual Workbench (Stewart and Associates, Madison, WI, USA; http://www.athenavisual.com) was used for parameter estimation.

3. Results and discussion

All inactivation experiments were conducted without stirring except the initial phase of 10 s since it was found previously that the GA inactivation at lower temperatures was strongly accelerated by stirring [18]. One obvious cause of the activity loss in stirred devices has traditionally been considered generated shear stresses. Maa and Hsu, however, demonstrated that

proteins could withstand the shear rates as high as 26,000 s^{-1} with a minimal conformation change [19]. A more probable cause of activity loss in stirred solutions is thus the enlargement of surface area and dynamic break-up and formation of air–liquid interface [20,21]. A linear relationship between the interfacial surface area and inactivation rate constant has been shown by Caussette et al. [22] in inactivation experiments where nitrogen was bubbled through an enzyme solution which was not stirred mechanically. On the contrary, a separation of air and enzyme solution by a layer of an immiscible liquid, hexadecane, led to a significant deceleration of GA activity loss [18]. It was further shown that the contribution of the effect of liquid–air interface became negligible at elevated temperatures (approximately above 45 °C) when the thermal effect became dominant [18].

3.1. Effect of enzyme concentration

The first series of experiments was conducted in the temperature range from 25 to 75 °C at three different initial enzyme concentrations. The ratio of the largest and smallest values of the concentration was about 33. Fig. 1a illustrates the inactivation measurements at 65 °C. It was found that the decrease of relative activity was strongly concentration-dependent at low temperatures (data not shown). The relative rate of inactivation was faster at lower enzyme concentrations, which could be explained by the adsorption saturation effect of the air–liquid interface. On the other hand, the relative rate of inactivation at elevated temperatures, where the influence of air–liquid interface was negligible, was independent of the enzyme concentration (Fig. 1b). This confirmed the absence of association reactions in the temperature range investigated.

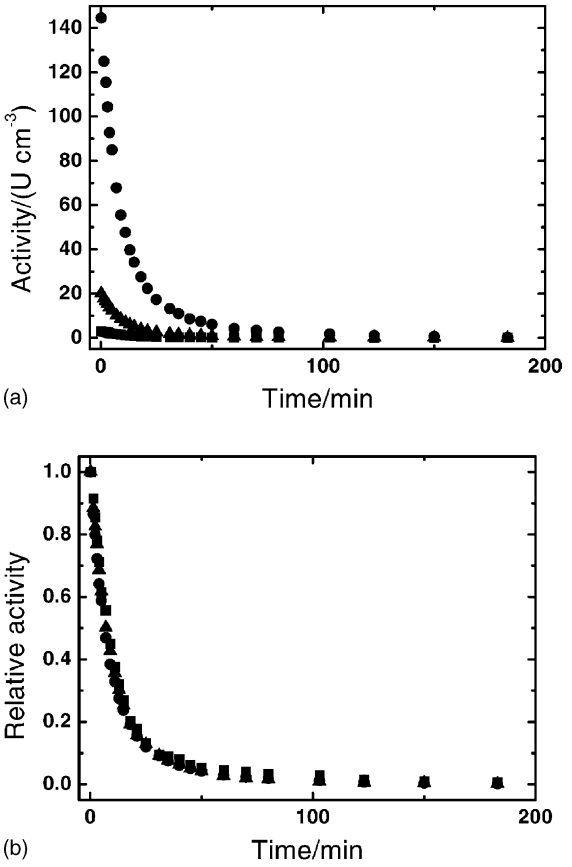

Fig. 1. Thermal inactivation of GA at pH 5.3 in a 0.05 mol dm^{-3} acetate buffer at 65 °C and initial enzyme concentrations of 1.2×10^{-4} (circles), 6.1×10^{-4} (triangles), and 4.0×10^{-3} g dm^{-3} (squares). (a) Activity vs. time; (b) relative activity vs. time.

3.2. Isothermal evaluation

3.2.1. First-order model

The above mentioned series of experiments was used to design an experimental plan with the following requirements: a temperature range covering the whole region of thermal inactivation from very slow to very fast; a sufficient number of temperature values in the chosen interval so that the influence of different phases of thermal inactivation could be exhibited and consequently identified; and frequent monitoring of activity loss (20–30 points at each activity curve) until its decrease essentially to nil.

Since the evaluation of above mentioned experiments showed that the highest accuracy of inactivation experiments was achieved at the initial enzyme concentration of 4.0 mg dm^{-3}, this single value was further applied in this study. The inactivation experiments were made in the temperature range of 48–76 °C with an increment of 4 °C (Fig. 2a–d). An usual first step in analysing the kinetics of inactivation of any enzyme is to check the suitability of first-order kinetics. This is often done graphically by plotting the data in a semi-logarithmic dependence of activity on time. We have made a quantitative evaluation using

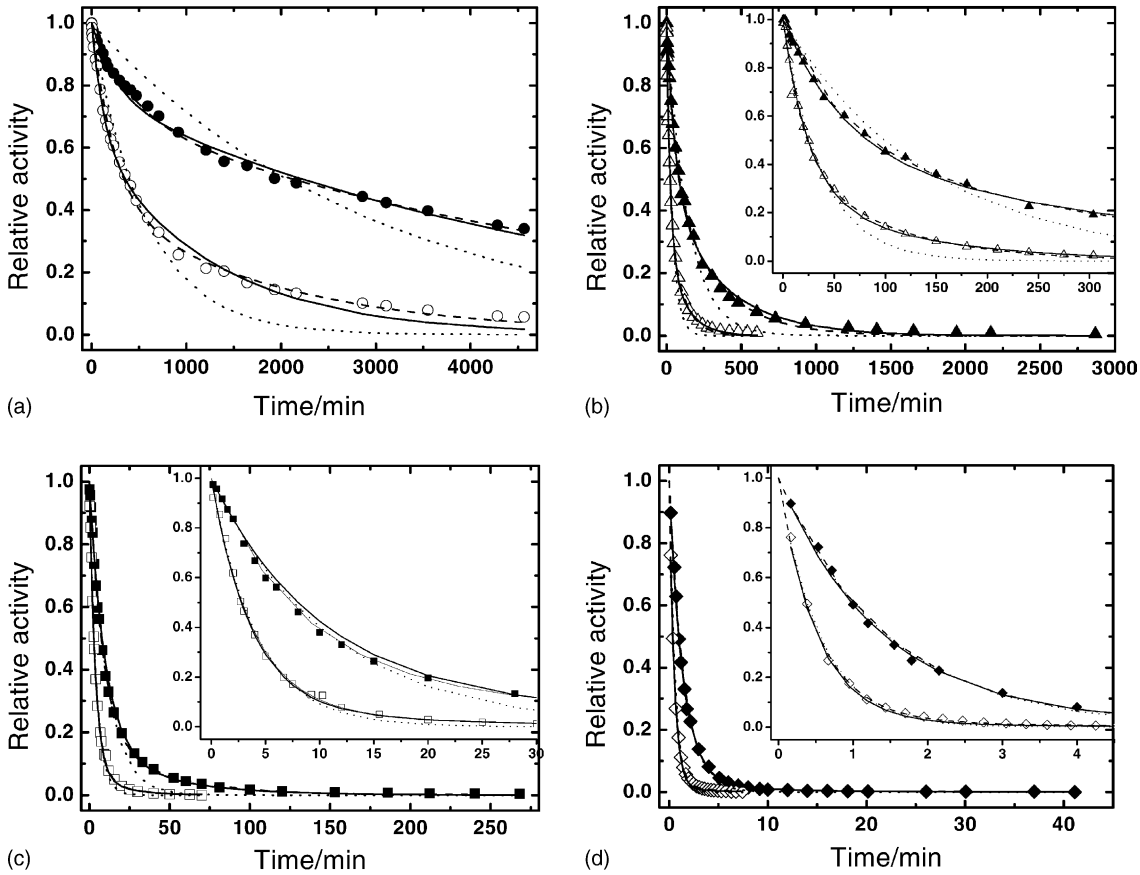

Fig. 2. Thermal inactivation of GA at pH 5.3 in a 0.05 mol dm^{-3} acetate buffer at 65 °C and initial enzyme concentrations of 4.0×10^{-3} g dm^{-3}. (a) 48 °C (closed circles), 52 °C (open circles); (b) 56 °C (closed triangles), 60 °C (open triangles); (c) 64 °C (closed squares), 68 °C (open squares); (d) 72 °C (closed diamonds), 76 °C (open diamonds). The lines represent the fits using first-order model (dotted lines), bi-exponential model (dashed lines) and multitemperature Lumry–Eyring model (solid lines). The inset graphs in Fig. 2b–d represent approximately the first tenth of the time courses.

the relationship,

$$a = \frac{A}{A_0} = \mathrm{e}^{-kt} \quad (1)$$

where a is the relative enzyme activity, which is the ratio of immediate, A and initial, A_0 values of enzyme activity, k is the first-order rate constant, and t is the time. The fitted curves are plotted in Fig. 2a–d as dotted lines. The largest disagreement of the fitted curve with the experimental data is shown at 48 °C where the so-called biphasic inactivation is manifested by different trends of measured and calculated values in the whole time course. On the contrary, no visible discrepancy can be observed at 68 °C and higher temperatures. In the intermediate values of temperature, from 52 to 64 °C, the differences are apparent mainly in the second phase of process when, with increasing temperature, they become smaller and appear at lower residual activities. For example, at 60 °C, this disagreement was observed at the values of the relative activity below 0.1. These observations also explain why other authors failed to identify the biphasic character of GA inactivation. The reasons are the lack of sufficient data at low residual activities and missing measurements at low temperatures.

Table 1 presents the values of residual sum of squares for the first-order model at each temperature. They decreased from the value of 1410 at 48 °C to the

Table 1
Comparison of evaluation of experimental data of GA inactivation using different approaches

Temperature (°C)	n	$\xi^2 \times 10^4$		
		1	2	3
48	30	1410	41.6	75.5
52	31	1372	75.0	159.8
56	31	547	31.7	46.3
60	28	521	108.1	135.1
64	28	207	15.9	104.6
68	21	83.0	50.7	57.1
72	25	38.9	29.6	31.3
76	23	27.9	16.6	28.3

The table columns give the values of the residual sum of squares, ξ^2, at individual temperatures with n experimental activity data. (1) First-order model, (2) bi-exponential model, (3) Lumry–Eyring model by the multitemperature fit.

value of 27.9 at 76 °C. The corresponding mean errors of relative activity were 0.070 and 0.011, respectively, which confirmed the unsuitability of first-order kinetics to describe comprehensively the inactivation of GA.

3.2.2. Bi-exponential models

A bi-exponential relationship,

$$a = \alpha e^{-k_1 t} + (1-\alpha)e^{-k_2 t} \quad (2)$$

was further used to smooth the experimental data. The results presented in Table 1 confirm that very good fits were achieved using Eq. (2). The residual sums of squares were independent of temperature and varied from 15.9 to 108.1. The corresponding mean errors of relative activity were then in the interval of 0.008–0.021. The calculated activity courses (dashed lines in Fig. 2a–d) demonstrate that the fits were very close and exhibited no systematic deviations. The average deviation of measured and calculated activities evaluated through all temperatures is 1.4% of the initial activity.

3.2.2.1. Isozyme model. As has been shown previously, the bi-exponential equation corresponds to different simple mechanisms of enzyme inactivation [9] such as the Lumry–Eyring mechanism of a two-step series reaction [23] or the so-called isozyme mechanism (Eq. (3)), which is formed by two parallel one-step irreversible reactions of native isozyme forms N_1 and N_2 to irreversibly inactivated enzyme forms I_1 and I_2.

$$N_1 \xrightarrow{k_1} I_1, \qquad N_2 \xrightarrow{k_2} I_2 \quad (3)$$

The rate constants, k_1 and k_2, of the isozyme mechanism are identical with the exponents in Eq. (2). The parameter α in Eq. (2) corresponds to the initial molar fraction of more labile isozyme. The isozyme mechanism can thus be very easily linked to the bi-exponential relationship of activity on time represented by Eq. (2). Fortunately, its consistency can be easily checked if inactivation measurements are available at different temperatures. Fig. 3 shows that the parameter α had changed with temperature. This is, however, inconsistent with the isozyme mechanism since the initial distribution of isozyme forms must be constant. The biphasic inactivation of GA in this study cannot be thus explained by a significantly different inactivation behaviour of the GA1 and GA2 isozymes which is in agreement with the inactivation experiments performed with isolated isozymes [5].

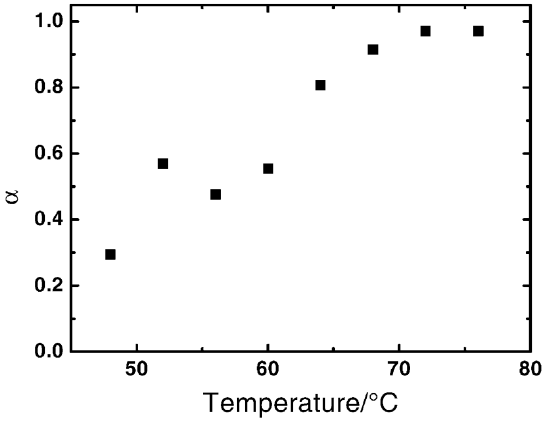

Fig. 3. Temperature dependence of the parameter α in Eq. (2).

3.2.2.2. Lumry–Eyring model. As has been mentioned above, Eq. (2) corresponds also to the integrated form of a model derived from the Lumry–Eyring mechanism,

$$N \underset{k_{1-}}{\overset{k_{1+}}{\rightleftarrows}} D \xrightarrow{k_2} I \quad (4)$$

where the native form N is reversibly transformed to the denatured form D which reacts further

irreversibly to the form I. The Lumry–Eyring's concept is almost exclusively used in the form that the first reaction is considered to be a very fast equilibrium reaction [2,13]. This assumption leads to a simple exponential relationship of activity on time. We, however, applied the Lumry–Eyring mechanism without this limitation so that the first step could have a kinetic character.

The integrated form of the Lumry–Eyring model can be derived from the mechanism by applying the mass action principle. An inactivation process can be described by a set of the following differential equations,

$$\frac{dc_N}{dt} = -k_{1+}c_N + k_{1-}c_D \quad (5a)$$

$$\frac{dc_D}{dt} = k_{1+}c_N - k_{1-}c_D - k_2c_D \quad (5b)$$

$$t = 0, \qquad c_N = c_{N0}, \qquad c_D = 0 \quad (5c)$$

The relative activity, a, is then simply calculated from the equation,

$$a = \frac{c_N}{c_{N0}} \quad (6)$$

The analytical solution of Eqs. (5) and (6) yields quite a complicated algebraic expression [24] therefore we estimated the rate constants from the differential equations using the parameter estimation procedures of the software Athena Visual Workbench.

As has been mentioned above, the isothermal evaluation always provides the same values of residual sum of squares for the isozyme and Lumry–Eyring models. Unlike the isozyme model, the Lumry–Eyring model does not contain any intrinsic control of its correctness. It could be only applied a check of compliance of the temperature dependence of calculated rate constants with an exponential function. Large random fluctuations of rate constants with temperature can question the validity of applied model [9]. Fig. 4 shows that such fluctuations were present only at the rate constant k_{1-}. But, the other two rate constants, which had dominant effect on the activity loss in the most part of the investigated temperature interval, exhibited a surprisingly close agreement with an exponential relationship on reciprocal temperature.

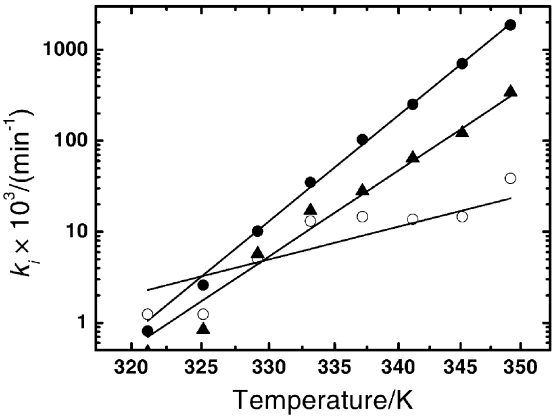

Fig. 4. Arrhenius plots (with reciprocal horizontal and logarithmic vertical axes) of the temperature dependence of the rate constants of the Lumry–Eyring model. The points represent the values obtained by isothermal evaluation, k_{1+} (closed circles), k_{1-} (open circles), and k_2 (triangles). The solid lines represent the courses calculated from the coefficients of the Arrhenius equation obtained by the multitemperature evaluation.

3.3. Multitemperature evaluation

Since the isothermal evaluation did not disqualify the Lumry–Eyring model, this was tested also by the multitemperature evaluation [10,11]. The model described above by Eqs. (5) and (6) was modified by including the equations expressing the temperature dependence of the rate constants in the form of a rearranged Arrhenius equation,

$$k_i = e^{\ln k_{i0}} e^{[E_i/RT_0(1-T_0/T)]} \qquad i = 1+,\ 1-,\ 2 \quad (7)$$

where E_i is the activation energy of the ith reaction, R is the universal gas constant, and k_{i0} the value of the rate constant k_i at the reference temperature T_0 (333.15 K in this study). An alternative to the Arrhenius equation is an equation derived from the Eyring transition state theory yielding activation enthalpies and entropies [13]. Both approaches give, however, the same quantitative description of experimental data and differ only in interpretations of their parameters.

A simultaneous evaluation of all experimental data of GA inactivation provided a very good fit as illustrated in Fig. 2a–d. The largest deviations between the predicted and experimental courses can be seen at 52 °C. This is confirmed also by the value of the residual sum of squares equal to 159.8 presented in

Table 2
The 95% confidence intervals of the parameters of the Lumry–Eyring model applied to the thermal inactivation of GA in the temperature interval of 48–76 °C

Parameter	95% Confidence interval
$k_{1+,0}/(\text{min}^{-1})$	$(3.09 \pm 0.09) \times 10^{-2}$
$k_{1-,0}/(\text{min}^{-1})$	$(6.50 \pm 1.47) \times 10^{-3}$
$k_{2,0}/(\text{min}^{-1})$	$(1.08 \pm 0.27) \times 10^{-2}$
$E_{a,1+}/(\text{kJ mol}^{-1})$	250 ± 3
$E_{a,1-}/(\text{kJ mol}^{-1})$	77 ± 18
$E_{a,2}/(\text{kJ mol}^{-1})$	203 ± 24

Table 1. This value corresponds to the mean error of relative activity of 0.024 that can be still considered a good agreement. Table 1 further shows that there is no systematic trend in the residuals with temperature.

The average deviation of measured and calculated activities evaluated through all temperatures was 1.7% of the initial activity. If the value of 1.4% is considered as a representation of the true error of the experimental procedure, the increase to the value of 1.7% due to the lack of fit can be considered acceptable and the model derived from the Lumry–Eyring mechanism can be accepted as adequate for the description of the kinetics of GA inactivation. Table 2 presents the values of estimated parameters of the model together with their 95% confidence intervals. The accuracies of the estimated parameters are, in general, very good when the highest accuracies are associated with the parameters of the forward reaction of the first step and the lowest accuracies with the backward reaction of the same step.

These parameter values were used to plot the calculated dependences of the rate constants (Fig. 4) and equilibrium constant of the first reaction (Fig. 5) on temperature. These figures show that, between 48 and 76 °C, the rate constant of the unfolding reaction increased by three orders of magnitude whereas the rate constant of refolding only approximately 10 times. It is further interesting to compare the rate constants of the second and first, backward reactions. Fig. 4 shows that their Arrhenius plots crossed at about 55 °C and, above this threshold, the rate constant of the second, irreversible reaction was significantly larger. This indicates that the reversible character of inactivation might be suppressed already at rather low temperatures. The destabilizing effect of temperature is further illustrated in Fig. 5. The values of the equilibrium constant show that the equilibrium of the reversible reaction was

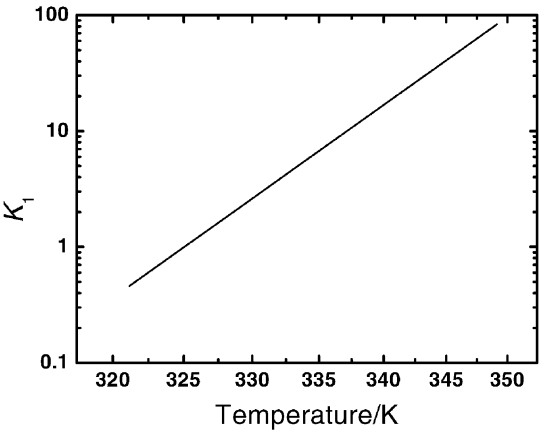

Fig. 5. The calculated temperature dependence of the equilibrium constant of the Lumry–Eyring model. The plot has reciprocal horizontal and logarithmic vertical axes.

shifted towards the native form below 50 °C whereas, above 75 °C, the native form would be present in negligible amounts in equilibrium conditions.

Different values of activation energies of GA inactivation could be found in the studies of other authors [2,4] but none of them was at the pH used in the present study. Due to a strong dependence of the kinetics of GA inactivation on pH, the only reasonable values which can be compared with ours are the values reported for the unfolding of GA at pH 6.0 in MES buffer by Christensen et al. [2]. They estimated the values of activation energies to be 253 kJ mol^{-1} for the one-step irreversible unfolding of GA1 and 208 and 289 kJ mol^{-1}, respectively, for the two-step irreversible unfolding of GA2. These values, obtained by differential scanning calorimetry, show quite an interesting quantitative correspondence with our values in Table 2 (250 versus 203, respectively, for the forward reactions). One must be, of course, very careful with making conclusions on this coincidence. Nevertheless, this could imply that individual steps of GA inactivation could be associated with large rearrangements of protein structure during unfolding.

4. Conclusions

It was shown that glucoamylase in the investigated temperature range lost activity primarily by a denaturation pathway when association/aggregation

bimolecular steps were not significant. A two-step mechanism of inactivation was identified as necessary to describe the kinetics. The isozyme model of kinetics was not identified which suggests that the inactivation kinetics of both isozyme forms is probably very similar. The method of multitemperature evaluation confirmed that a consistent description of the experimental data in the whole temperature range could be achieved using the Lumry–Eyring mechanism. The evaluated parameters confirmed a strong destabilizing effect of temperature that has a strongly irreversible character. The calculated activation energies had values comparable with those typical for denaturation processes. The results obtained are a good starting point for the investigation of the GA inactivation at the conditions of starch hydrolysis where the presence of starch and products of its degradation influence the enzyme stability.

Acknowledgements

This work was supported by the Polish KBN Grant No. 3T09C 03119 and Slovak Grant Agency for Science VEGA Grant No. 1/7343/20. The first author is grateful to the Deutsche Forschungsgemeinschaft for the travel grant which allowed him to participate and present this study at the BioTrans 2001 conference held in Darmstadt, Germany on 2–7 September 2001. Gist-Brocades are kindly acknowledged for the gift of enzyme.

References

[1] C. Akerberg, G. Zacchi, N. Torto, L. Gorton, J. Chem. Technol. Biotechnol. 75 (2000) 306.
[2] T. Christensen, B. Svensson, B.W. Sigurskjold, Biochemistry 38 (1999) 6300.
[3] Z. Sasvári, B. Asbóth, Enzyme Microb. Technol. 22 (1998) 466.
[4] M. Przybyt, H. Sugier, Starch/Stärke 40 (1988) 171.
[5] E. Várallyay, Z. Sasvári, A. Hoschke, B. Asbóth, Acta Alimentaria 23 (1994) 93.
[6] T. Bahar, S.S. Celebi, Enzyme Microb. Technol. 23 (1998) 301.
[7] M.Y. Arica, H. Yavuz, S. Patir, A. Denizli, J. Mol. Catal. B-Enzymatic 11 (2000) 127.
[8] B. Szajáni, G. Klamár, L. Ludvig, Enzyme Microb. Technol. 7 (1985) 488.
[9] M. Polakovič, P. Vrábel, Process Biochem. 31 (1996) 787.
[10] P. Vrábel, M. Polakovič, V. Štefuca, V. Báleš, Enzyme Microb. Technol. 20 (1997) 348.
[11] P. Vrábel, M. Polakovič, Š. Godó, V. Báleš, P. Dočolomanský, P. Gemeiner, Enzyme Microb. Technol. 21 (1997) 196.
[12] F.X. Malcata, C.G. Hill, C.H. Amundson, Biotechnol. Bioeng. 39 (1992) 1097.
[13] E.P. Schokker, M.A.J.S. Vanboekel, J. Agric. Food Chem. 45 (1997) 4740.
[14] L.M.M. Tijskens, P.S. Rodis, M.L.A.T.M. Hertog, U. Kalantzi, C. van Dijk, J. Food Eng. 35 (1998) 111.
[15] M. Boy, A. Dominik, H. Voss, Process Biochem. 34 (1999) 535.
[16] M.M. Tijskens, R. Greiner, E.S.A. Biekman, U. Konietzny, Biotechnol. Bioeng. 72 (2001) 323.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[18] J. Bryjak, Inzynieria Chemiczna i Procesowa 22 (2001) 295.
[19] Y.F. Maa, C.C. Hsu, Biotechnol. Bioeng. 51 (1996) 458.
[20] Y.F. Maa, C.C. Hsu, Biotechnol. Bioeng. 54 (1997) 503.
[21] S. Colombie, A. Gaunand, B. Lindet, Enzyme Microb. Technol. 28 (2001) 820.
[22] M. Caussette, A. Gaunand, H. Planche, S. Colombie, P. Monsan, B. Lindet, Enzyme Microb. Technol. 24 (1999) 412.
[23] R. Lumry, H. Eyring, J. Phys. Chem. 58 (1954) 110.
[24] A. Sadana, Biocatalysis. Fundamentals of Enzyme Deactivation Kinetics, Prentice Hall, Englewood Cliffs, NJ, 1991.