Analysis of the Inactivation Kinetics of Freeze-dried α-Amylase from *Bacillus amyloliquefaciens* at Different Moisture Contents

J. Saraiva, J. C. Oliveira*, M. Hendrickx, F. A. R. Oliveira and P. Tobback

J. Saraiva, J. C. Oliveira, F. A. R. Oliveira: Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R.
Dr António Bernardino de Almeida, 4200 Porto (Portugal)
M. Hendrickx, P. Tobback: Center for Food Science and Technology, Unit of Food Preservation, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B3001 Heverlee (Belgium)

The thermal inactivation kinetics of freeze-dried α-amylase in a solid matrix was studied at water contents ranging from 1.5 to 23.9 g water per *100 g dry solid. These conditions were obtained by equilibration in dry environments, with water activities ranging between 0.11 and 0.88. Isothermal inactivation experiments in the range 135 to 150 °C were performed. Results were analysed with both the Bigelow and Arrhenius models. It was concluded that there was no statistical significance to suggest that the water content influenced the kinetic parameters. An activation energy of 128 kJ/mol and pre-exponential factor with a logarithm of 33.9 described all the results well.*

Introduction

It is known that dehydrated enzymes can withstand temperatures in the range of 100 to 150 °C (1–5). For applications where enzymes need to be strongly thermally stable, dried enzymes are a good choice. This is the case in the development of enzyme based time–temperature integrators (TTIs) to assess thermal processes at sterilization temperatures (100 to 140 °C). The fundamental problem in developing such a system is to obtain the necessary inactivation kinetic parameters to monitor a certain aspect. For thermal processing of low-acid foods, the evaluation of microbial lethality requires a z-value of 10°C (6) and an adequate D-value for accurate measurements. To be effective at temperatures higher than 100°C (sterilization), the TTI system has to be very stable, i.e. the enzyme used must remain partially active after the heat process.

Developing a TTI system implies accurately describing the kinetic behaviour of the system. The correct interpretation of inactivation kinetics depends both on obtaining good experimental data and on their critical interpretation. For first-order decay, two models are widely used: the Bigelow model, using D and z factors, more usual in thermal processing and microbiology, and the Arrhenius model, using the pre-exponential factor (k_o) and activation energy (E_a) concepts, commonly used in chemical kinetics. Kinetic parameters obtained with one model should not be directly converted to those of the other (7,8).

α-Amylases [(1→4) glucan 4-glucanohydrolases, EC 3.2.1.1.] catalyse the breaking of α-1,4-glucosidic bonds in amylose and amylopectin molecules (9). This type of enzyme is widely used in the sugar, textile and brewery industries, one of its most important industrial applications concerning the liquefaction of starch to oligossacharides for subsequent production of glucose syrups by glucoamylases. Starch liquefaction must take place at temperatures higher than the gelatinization temperature, and the use of highly thermostable α-amylases is therefore required. The search for new or improved heat stable α-amylases has been the subject of numerous works reported in literature (10,11).

The thermal stability of solid-phase α-amylase from *Bacillus amyloliquefaciens* has not yet been studied. In order to evaluate the possibility of using this enzyme to develop a TTI to work between 100 and 140 °C its irreversible thermal inactivation was studied as a function of water content.

Materials and Methods

Lyophilization and equilibration

α-Amylase from *B. amyloliquefaciens* (EC 3.2.1.1., Sigma Co., St Louis, Mo 63158, U.S.A. — source confirmed by the supplier) was lyophilized (130 g/L) in small vials in a freeze-drier Secfroid (Lausanne, Swit-

zerland). The lyophilized enzyme was equilibrated for 6 d at 4°C in hermetically closed jars above the following saturated salt solutions: lithium chloride, potassium acetate, potassium carbonate, sodium chloride, ammonium sulphate and potassium chloride, which establish the water activities (a_w) of 0.11, 0.23, 0.43, 0.76, 0.82 and 0.88, respectively (12). Each equilibration establishes a given water content in the enzyme, corresponding to that in equilibrium over the saturated salt solutions. The sorption isotherm was reported previously (13) and the water contents corresponding to the six water activities reported here were, respectively, 1.48, 2.64, 4.92, 16.02, 18.53 and 23.89 g per 100 g dry solid.

Thermal treatment

For each water content, the vials containing the enzyme powder were tightly closed prior to the inactivation experiments using aluminium caps and a hand crimper from Chrompack International BV (Middelburg, The Netherlands). This step was performed within about 5 s to avoid atmosphere changes. The closed vials were transferred directly from 4°C to an oil bath previously stabilized at the temperature specified for the experiment. At predetermined time intervals, the vials were quickly transferred to an ice bath. After cooling, the vials were opened, 0.625 or 0.5 mL (depending on which procedure was used for the amylase activity analysis) of Tris-HCl buffer (pH 7.2; 0.1 mol/L) was added to each and they were kept in the ice bath until analysis of residual activity. Experiments were duplicated at each temperature for each water content. Since the heating times were significant and the vials containing the enzyme powder very small, thermal lag was neglected.

Activity analysis

The α-amylase activity was measured according to procedure No. 576 or No. 577 of Sigma Diagnostics (both procedures are based on the hydrolysis of α-1,4 glucosidic bonds by amylase in *p*-nitrophenyl-α-D-maltoheptaoside, releasing *p*-nitrophenol, which absorbs maximally at 405 nm). The increase in optical density (O.D.) at 405 nm was recorded (LKB 4053 Kinetics Spectrophotometer, Cambridge, U.K.), and linear regression was used to calculate the initial reaction rate at 30°C (ΔOD/min), which was used to express enzyme activity.

Data analysis

The inactivation kinetics of amylase were analysed, considering a first-order reaction rate. For a constant temperature:

$$\frac{A}{A_0} = \exp(-k \times t) \qquad \text{Eqn [1]}$$

where A = total residual activity of the enzyme (ΔO.D./min), A_0 = total initial activity of the enzyme (ΔO.D./min), k = rate constant (min^{-1}) and t = time (min).

The D-values were calculated according to the definition:

$$k = \frac{\mathrm{Ln}(10)}{\mathrm{D}} \qquad \text{Eqn [2]}$$

where D = decimal reduction time (min).

For the Bigelow model, rate constants are considered to vary exponentially with temperature:

$$\mathrm{D} = \mathrm{D_r} \times 10^{\frac{T_r - T}{z}} \qquad \text{Eqn [3]}$$

where D_r = decimal reduction time at a reference temperature (min), T = temperature (K), T_r = a reference temperature (K), z = z value (°C or K), defined as the temperature increase needed to reduce the D-value by one log unit. The reference temperature used was 140°C (413 K).

For the Arrhenius model, the rate constant is considered to vary exponentially with the reciprocal of temperature:

$$k = k_r \times \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right)\right) \qquad \text{Eqn [4]}$$

where k = rate constant at temperature T (min^{-1}), k_r = rate constant at a reference temperature (min^{-1}), T = temperature (K), T_r = reference temperature (K), E_a = activation energy (J/mol) and R = gas constant (8.314 J/(mol.K)).

From Eqns [2–4] it follows that:

$$E_a = \frac{R \times T \times T_r \times \ln 10}{z} \qquad \text{Eqn [5]}$$

This equation is only valid if T_r is the same for both models.

In chemical kinetics, the Arrhenius model is usually applied for an infinite reference temperature:

$$k = k_0 \exp\left(\frac{E_a}{R \times T}\right) \qquad \text{Eqn [6]}$$

with k_0 being the pre-exponential factor. In that case, conversion between E_a and z cannot be obtained directly. Datta (7), following a linearization around the T_r of the Bigelow model, also obtained Eqn [5], which is exact only at the linearization point. Equation [5] is useful because it highlights the basic difference of the two models: if one is reality, the other suggests a parameter varying linearly with temperature. However,

for the temperature range usually relevant in food processing, it is normally impossible to distinguish between them, both providing equally good fits.

There are two methods for estimating the model parameters in both cases: one-step and two-step. With the one-step method, all inactivation data are fitted to Eqn [1], after applying Eqn [3] (or [4]) to express the temperature dependency of the rate constant, using a non linear regression. The two-step method consists of fitting the results of each isothermal experiment with Eqn [1] to obtain rate constants at different temperatures, which are then fitted to [3] (or [4]). Logarithmic forms of the equations allow for the use of linear regressions in the two steps. This second method is more commonly found in literature, although the first one should be preferred because confidence regions are much smaller and the statistical parameters are straightforward to interpret, there being only one regression.

Statistical analysis

The kinetic model has two parameters, both with a given uncertainty. Each has its individual interval of confidence, but these are not the best way to describe uncertainty in a two-dimensional reality.

The solution obtained with a one-step regression is the pair of values that gives the minimum residual between the model predictions and the experimental points. The confidence region in a two-dimensional space is defined by the area containing all pairs of parameters that yield a residual that is not significantly different from the minimum. The boundary of this region is defined by the sum of squares of the residuals, given by the following equation, for the confidence level specified:

$$SS_{y\%} = SS_s \times \left[1 + \frac{P}{N-P} \times F_y\,(P, N-P)\right] \qquad \text{Eqn [7]}$$

where $SS_y\%$ = sum of squares of residuals at y% confidence level, SS_s = sum of squares of residuals of the estimated solution, P = number of parameters (in this case 2), N = number of experimental points and $F_y(P,N–P)$ = value of the F distribution for P and N–P.

The 90% joint confidence level is the most usual, because the limits of 90% joint confidence regions are close to the individual 95% confidence intervals (14) (the probability of simultaneous occurrence of two events, each with an individual probability of 95%, is about 90%). Therefore, when analysing results with a two-parameter model, this joint confidence region should be determined for interpreting the possible variability arising from the experimental error, rather than using just the individual confidence intervals. This aspect will be very well shown in this work.

A simple least squares, nonweighted residual was used. A FORTRAN program was written to determine the 90% confidence region. The increment step used to determine the isoresidual lines was 0.5 (min and°C or kJ/mol). A pair of parameters immediately before and after the 90% confidence region border was determined and the border calculated as the average of the two pairs, which means that the maximum uncertainty in its determination is 0.25. The shape of a joint confidence region strongly depends on the type of model, and therefore the Arrhenius model with infinite reference temperature (Eqn [5]) was used, since it leads to the greatest difference in relation to the Bigelow model.

Results and Discussion

Verification of initial assumptions

Preliminary experiments indicated that the activity increased linearly with enzyme concentration up to 0.70 ΔO.D./min and 0.33 ΔO.D./min for procedures No. 576 and 577, respectively. The experimental samples always fell in these ranges.

Before performing the inactivation studies, the stability of amylase during lyophilization and stability above saturated salt solutions were analysed. The residual activity after lyophilization was always higher than 95%. During equilibration at 4°C above saturated salt solutions, corresponding to water contents of 1.5, 4.9 and 23.9 g per 100 g dry solid, the enzyme activity varied less than 3% for up to 11 d of study.

The presence of certain cations (e.g. calcium and sodium) is reported in literature to affect the thermal characteristics of α-amylase in aqueous solutions (11,15), and a similar effect should not be excluded in the present case. For lyophilization, water was used instead of a buffer to avoid the effect of the buffer ions (on both water adsorption and inactivation behaviour).

Activity regeneration of thermally inactivated enzymes in aqueous solutions has been reported (e.g. peroxidase (16)). For inactivation of dried α-amylase, no activity regeneration was found after 24 h storage at 4°C.

Inactivation data

Table 1 shows the parameters obtained with the Bigelow model and their individual 95% errors, using the one-step procedure.

Figures 1 and **2** show typical results, for the experimental data obtained with water contents of 2.6 and 16.0 g water per 100 g dry solid and the model curves.

Table 1 Kinetic parameters for the Bigelow model obtained with the one-step method (means±95% confidence interval, for an average of 16 to 20 experimental points)

Water content (g per 100g dry solid)	$D^{140°C}$ (min)	z (°C)
1.5	60.7±3.1	28.2±3.1
2.6	66.4±3.7	25.2±2.1
4.9	65.6±2.7	30.0±2.3
16.0	69.5±2.7	24.1±1.0
18.5	60.2±2.8	27.1±2.4
23.9	72.0±4.9	21.2±2.1

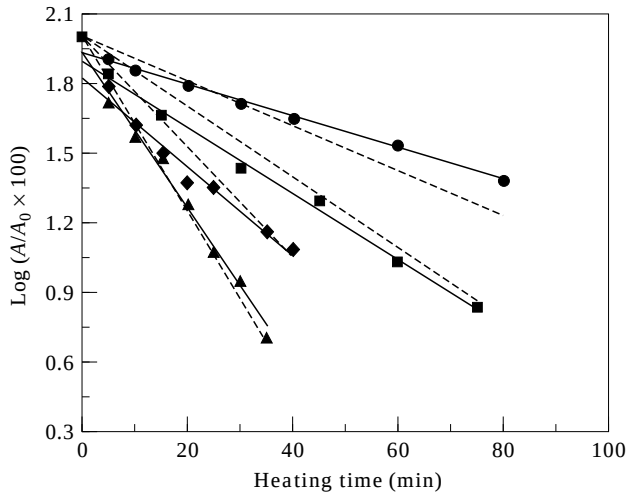

Fig. 1 Inactivation data of α-amylase with 2.6 g water per 100 g dry solid at 135°C (●), 140°C (■), 145°C (◆) and 150°C (▲), and the fits provided by the one-step (----) and two step(———) methods, with Bigelow's model

It should be noted that the activity decay of dehydrated α-amylase clearly follows the first-order model considered. For soluble and covalentely immobilised *B. licheniformis* α-amylase (at temperatures below 85°C), a biphasic inactivation profile was reported (11,17). A first-order inactivation kinetic was described for soluble α-amylase from *B. amyloliquefaciens* (at 90°C (10,18) and at temperatures between 70 and 85°C (19)).

The z-value of amylase (see **Table 1**) is not as sensitive to water content as in the case of peroxidase (5), but the lowest value for both proteins occurs for the highest moisture content: the z-value for peroxidase increased from 18°C to 32°C and then decreased steeply to 17°C at 0.88 a_w. Results similar to those obtained for peroxidase were reported for wheat grain ribonuclease (2). Meerdink and van't Riet (20) reported the activation energy as being an exponentially increasing function of the moisture content for α-amylase thermal

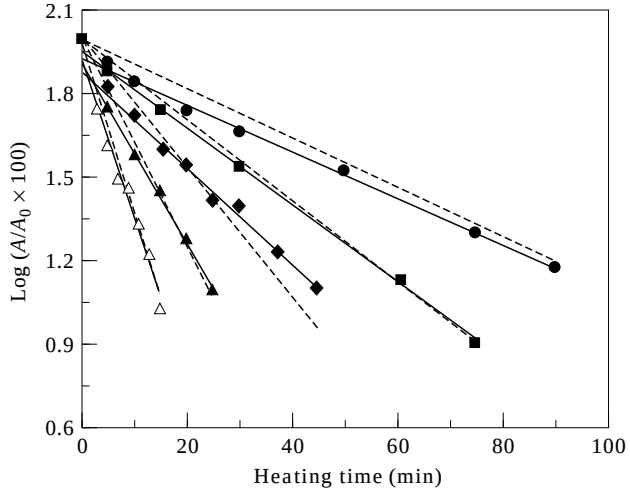

Fig. 2 Inactivation data of α-amylase with 16.0 g water per 100 g dry solid at 135°C (●), 140°C (■), 145°C (◆), 150°C (▲) and 155°C (△), and the fits provided by the one-step (----) and two-step (———) methods, with Bigelow's model

inactivation from *B. licheniformis* during drying, but they worked at much higher moisture contents and used maltodextrins as a support material, which makes comparisons difficult. As far as it is known, no data concerning the inactivation of solid-phase α-amylase from any source have yet been reported, and no comparisons can be made. The variation profile of the z-value of dried α-amylase from *B. amyloliquefaciens* with water content seems to be 'random' and therefore less predictable than for peroxidase. The dried *B. amyloliquefaciens* α-amylase lyophilized from water clearly shows less sensitivity to temperature than in aqueous buffer solution, which is revealed by the z-value of 7°C obtained for its inactivation in aqueous Tris-HCl buffer (0.1 mol/L), at pH 8.5 (21). Although the temperature range of inactivation is about the same for the α-amylase studied in this work and for horseradish peroxidase (5), the former is slightly less stable than the latter, with D-values at 140°C ranging from 60 to 70 min for amylase and from 120 to 200 min for peroxidase.

Some further comments should be made at this point regarding enzyme stability. The temperature range of inactivation (135 to 150°C) is much higher when compared with inactivation in buffer aqueous solution (the enzyme rapidly inactivates at 90°C) (18). Even for more heat-resistant α-amylases from thermophilic organisms, such as *B. licheniformis*, the inactivation temperatures in solution are lower (90 to 100°C) (10). The fact that enzymes are generally found to be extremely stable when dried indicates that water has a fundamental role in the thermal inactivation of proteins and therefore that dehydration is a method of improving stabilization. This effect is certainly due to its participation in the inactivation mechanism. In aqueous buffer, inactivation of α-amylase from *B. amyloliquefaciens* is caused by a monomolecular conformational process (formation of incorrect structures) (18). Because the presence of water in protein increases its flexibility and allows thermal unfolding (22), the suppression of water should stabilize the enzyme. However, the inactivation mechanism of a dried form of the enzyme is not known, and other alternatives are possible.

Model evaluation

Bigelow model. **Figure 3** shows all the 90% joint confidence intervals for the six different water contents. The typical case for the water content of 18.5 g of water per 100 g dry solid is highlighted, to show that the two parameters have a low co-linearity and that the limits of the individual 95% confidence intervals are close to the limits of the 90% joint confidence region (this case is precisely the one in which the difference was greater; in other cases they were even closer). This results in the fact that most pairs of values for D_r and z between the interval $D_r \pm \Delta D$ and $z \pm \Delta z$, where Δ represents the 95% individual confidence error, generate a set of D-values at the various temperatures that model the

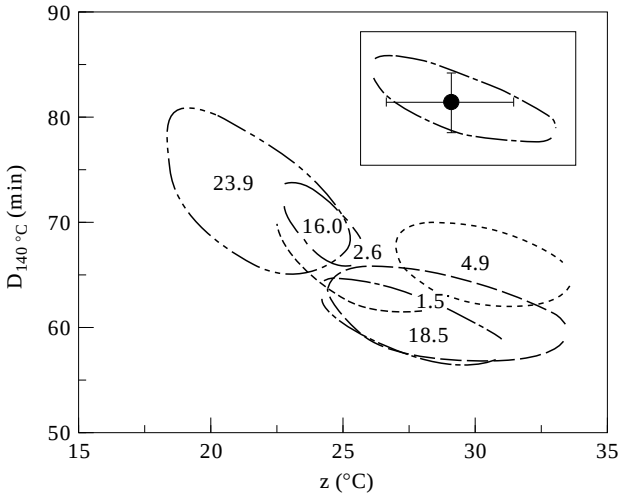

Fig. 3 90% joint confidence regions for all the water contents studied, for the Bigelow model. Water contents (g water per 100 g dry solid) are indicated in the graph, near the centre of their respective region. In the top right-hand corner box are the individual 95% confidence intervals for 18.5 g water per 100 g dry solid (the black circle indicates the solution)

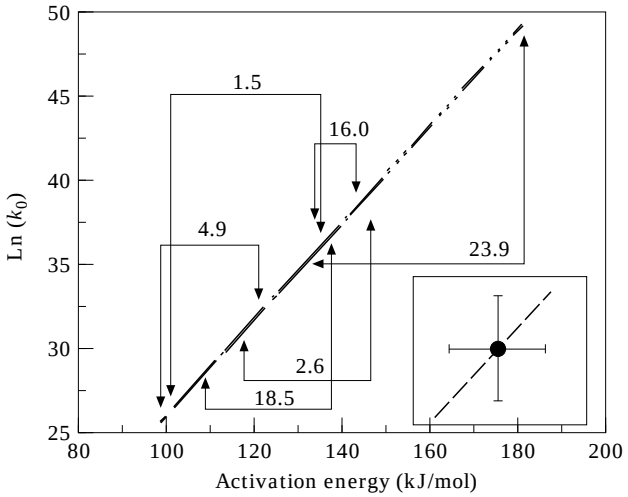

Fig. 4 90% joint confidence regions for all the water contents studied, for the Arrhenius model. The limits for each water content (g water per 100 g dry solid) are indicated in the graph. In the lower right-hand corner box, the individual 95% confidence intervals for 18.5 g water per 100 g dry solid are shown (the black circle indicates the solution)

whole set of experimental data with a residual smaller than the 90% residual.

Arrhenius model. **Table 2** shows the results obtained with the one-step method and the Arrhenius model. Once again, looking at these values, it would seem that although there is apparently some influence of the water content, there is no specific trend.

Figure 4 shows all the 90% joint confidence regions at the various water contents, and it can be seen that, with this model, there is a strong co-linearity between the two parameters. The 90% joint confidence region has consequently been squeezed down to a straight line. The individual confidence intervals for the same case as in **Fig. 3** (water content of 18.5 g water per 100 g dry solid) are also highlighted (lower right-hand corner box of **Fig. 4**). Although it is still true that the limits of the individual 95% confidence intervals are close to those of the 90% joint confidence region, there is only a very limited number of pairs of k_0 and E_a that can be used to generate model curves with a residual lower than the 90% residual. This graph clearly shows the importance of analysing the error region of the results. Users might consider that any pair of k_o and E_a within the $\ln k_0 \pm \Delta\ln k_0$ and $E_a \pm \Delta E_a$, with Δ being the 95% individual error range, would generate good predictions, but it can be seen in **Fig. 4** that some pairs (in the inverse inclination of the line of the 90% joint confidence region) would yield very bad predictions. The co-linearity of the parameters is so high that the whole confidence region for 18.5 g of water in 100 g enzyme water content could be fitted by a single straight line ($\ln k_0 = -3.15 + [0.289 \times E_a]$) with a correlation coefficient of 0.999. Moreover, all the confidence regions are segments of the same straight lines. Fitting all points of the different intervals in one single straight line yields:

$$\ln k = -(2.92 \pm 0.055) + (0.2878 \pm 0.00041) \times E_a \qquad \text{Eqn [8]}$$

This is a good description of the error region for all results, with the start- and end-points being the only difference between the different water contents: the slopes and the intercepts are the same. Furthermore, all regions overlap, except for 4.9 g of water per 100 g dry solid. For a highly co-linear region such as this, increasing the level of confidence from 90 to 95, 97.5 or 99% basically enlarges the joint confidence region by moving along the line. This means that if the result for 4.9 g of water per 100 g dry solid is considered to have been affected more by experimental error, we could consider that one single pair of k_0 and E_a might be used to fit all results.

The most adequate way to calculate such value is not by arithmetic average of individual results but by fitting the whole set of experimental points with one single regression. This procedure yielded $\ln k_0 = 33.9$ and $E_a = 128$ kJ/mol. Furthermore, individual rate constants estimated with these parameters should not be significantly different from the various individual ones obtained by the two-step method. The case in which the individual rate constant is more different from the one estimated with the global parameters is for the water

Table 2 Kinetic parameters for the Arrhenius model obtained with the one-step method (means±95% confidence interval, for an average of 16 to 20 experimental points)

Water content (g per 100g dry solid)	$\ln k_0$	E_a (kJ/mol)
1.5	31.0±3.7	117.7±12.0
2.6	35.1±3.1	132.2±10.8
4.9	28.9±2.4	110.7±8.3
16.0	37.2±1.8	139.5±6.4
18.5	32.7±3.1	123.4±10.9
23.9	41.8±4.5	155.5±15.8

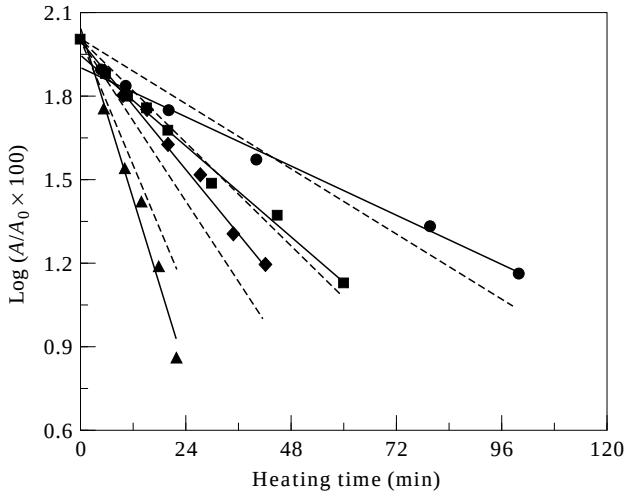

Fig. 5 Inactivation data of α-amylase with 23.9 g water per 100 g dry solid at 135°C (●), 140°C (■), 145°C (◆) and 150°C (▲), and the fits provided by the individual analysis (———) and by the average parameters (----) using the Arrhenius model

content of 23.9 g of water per 100 g dry solid at 150°C. This case was considered to be an outlier and not included in the determination of the global parameters (robust fitting). **Figure 5** shows the experimental points and the model predictions using the average kinetic parameters for this worst case. The larger error arises mainly from the final point of the outlier temperature. It can be seen that a larger deviation between model and experimental data results from the initial fall of the enzyme activity (particularly visible for the lowest temperature: 135°C).

It is apparent that there is no statistical significance to suggest that water content affects the kinetic behaviour of the enzyme, in the range tested, when analysing the Arrhenius model results.

Conclusions

The inactivation of freeze-dried α-amylase from *B. amyloliquefaciens* follows first-order decay kinetics. The inactivation temperatures (135 to 150°C) and the values obtained for the decimal reduction times clearly show that the enzyme is much more stable than in aqueous solution. Analysing the results with Bigelow's model (using a reference temperature of 140°C) indicates a small effect of water content on the z-value, which does not follow any particular trend. However, the analysis following the Arrhenius model (considering an infinite reference temperature) shows that there is no statistical significance to suggest that water content affects the enzyme stability in these ranges. A pre-exponential factor with a logarithm of 33.9 and an activation energy of 128 kJ/mol satisfactorily fitted all results. For a TTI development to assess sterilization processes, it is necessary to reduce the z-value to 10°C and decrease the stability of *B. amyloliquefaciens* α-amylase.

Acknowledgements

The authors would like to acknowledge the Commission of the European Communities, AAIR programme (AIR1-CT92-0746), for financial support. Jorge Saraiva acknowledges support from the Portuguese Junta Nacional de Investigação Científica (JNICT). The authors also wish to express their gratitude to Ronny Platteeuw for his help in the experimental work.

References

1 Zaks, A. and Klibanov, A.M. Enzymatic catalysis in organic media at 100°C. *Science*, **224,** 1249–1251 (1984)
2 Multon, J.L. and Guilbot, A. Water activity to the thermal inactivation of enzymatic proteins. In: Duckworth, R.B. (Ed.), *Water Relation of Foods*. London: Academic Press, pp. 379–396 (1975)
3 Rüegg, M., Moor, U. and Blanc, B. Hydration and thermal denaturation of β-lactoglobulin: a calorimetric study. *Biochimica et Biophysica Acta*, **400,** 334–342 (1975)
4 Hägerdal, B. and Martens, H. Influence of water content on the stability of myoglobin to heat treatment. *Journal of Food Science*, **41,** 933–937 (1976)
5 Hendrickx, M., Saraiva, J., Lyssens, J., Oliveira, J. and Tobback, P. The influence of water activity on thermal stability of horseradish peroxidase. *International Journal of Food Science and Technology*, **27,** 33–40 (1992)
6 Hayakawa, K.I. A critical review of mathematical procedures for determining proper heat sterilization process. *Food Technology*, **3,** 59–65 (1978)
7 Datta, A.K. Error estimates for approximate kinetic parameters used in food literature. *Journal of Food Engineering*, **18,** 181–201 (1993)
8 Nunes, R.V., Swartzel, K.R. and Ollis, D.F. Thermal evaluation of food processes: the role of a reference temperature. *Journal of Food Engineering*, **20,** 1–15 (1993)
9 Takagi, T., Tod, H. and Isemura, T. Bacterial and mold amylases. In: Boyer P.D. (Ed.), *The Enzymes*, 3rd Edn, Vol. V. New York: Academic Press, pp. 235–271 (1971)
10 Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H. and Udata, S. Amino acid residues stabilising a Bacillus α-amylase against irreversible thermoinactivation. *Journal of Biological Chemistry*, **264**(32), 18933–18938 (1989)
11 De Cordt, S. Vanhoof, K., Hu, J., Maesmans, G., Hendrickx, M. and Tobback, P. Thermostability of soluble and immobilised α-amylase from *Bacillus licheniformis*. *Biotechnology and Bioengineering*, **40,** 396–402 (1992)
12 Greenspan, L. Humidity fixed points of binary saturated aqueous solutions. *Journal of the Research of the National Bureau of Standards — A. Physics and Chemistry*, **81**A, 89–96 (1977)
13 Saraiva, J. Effect of environmental aspects on enzyme heat stability and its application in the development of time temperature integrators. PhD thesis, Escola Superior de Biotecnologia, Universidade Católica Portuguesa (1994)
14 Box, G.E.P., Hunter, W.G. and Hunter, J.S. *Statistics for Experimenters*. New York: John Wiley & Sons (1978)
15 Hasegawa, A. and Imahori, K. Studies on α-amylase. *Journal of Biochemistry*, **79**(3), 469–477 (1976)
16 Adams, J.B. The inactivation and regeneration of peroxidase in relation to the high temperature-short time processing of vegetables. *Journal of Food Technology*, **13,** 281–297 (1978)

17 VIOLET, M. AND MEUNIER, J.-C. Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase. *Biochemical Journal*, **263,** 665–670 (1989)
18 SUSAN, J. T. AND KLIBANOV, A. M. Mechanisms of irreversible thermal inactivation of Bacillus α-amylases. *Journal of Biological Chemistry*, **263**(7), 3086–3091 (1988)
19 DE CORDT, S., HENDRICKX, M., MAESMANS, G. AND TOBBACK, P. The influence of polyalcohols and carbohydrates on the thermostability of α-amylase. *Biotechnology and Bioengineering*, **43,** 107–114 (1994)
20 MEERDINK, G. AND VAN'T RIET, K. Inactivation of a thermostable α-amylase during drying. *Journal of Food Engineering*, **14,** 83–102 (1991)
21 DE CORDT, S., ÀVILA, I., HENDRICKX, M. AND TOBBACK, P. DSC and protein-based time-temperature-integrators: case study on α-amylase stabilized by polyols and/or sugar. *Biotechnology and Bioengineering*, **44,** 859–865 (1994)
22 ZAKS, A. AND KLIBANOV, A. M. The effect of water on enzyme action in organic media. *Journal of Biological Chemistry*, **263,** 8017–8021 (1988)