Thermal Inactivation Kinetics of Suspensions of *Bacillus amyloliquefaciens* α-Amylase in Hydrophobic Organic Solvents

J. Saraiva, J. Oliveira* and M. Hendrickx

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

The thermal inactivation of suspensions of α-amylase from Bacillus amyloliquefaciens *equilibrated at three low moisture contents and with added hydrophobic organic solvents of different hydrophobicity (dodecane, octane and 1-octanol) was systematically studied at temperatures between 135 to 150°C. The inactivation kinetics showed a first order decay in all cases. The enzyme is much more thermostable and less temperature sensitive than in aqueous solution. The behaviour was compared to inactivation in dry atmospheres, at similar water contents, without solvents. The organic solvents caused a larger influence of the water content and some environments caused significant changes in the rate constants, but the activation energy was not significantly affected. The solvent showing a higher impact on the kinetic parameters was 1-octanol.*

Introduction

Dehydration can be used to increase the thermal resistance of enzymes. An important application is the development of enzyme-based time–temperature integrators (TTI) to monitor sterilization processes. The increased resistance has been attributed to the role of water molecules in the inactivation mechanism (1).
Manipulating the thermal inactivation kinetics is essential for developing adequate TTI. A further possibility is the use of 'solvent engineering', i.e. modification of the environmental conditions of the protein using organic solvents in order to change the inactivation characteristics. Results reported in literature indicated that the stability of dried enzymes in organic solvents changed with water content and the nature of the organic solvent used (2,3). However, there are almost no data available in the literature concerning the effect of organic solvents on the temperature sensitivity of the inactivation rate (z value or activation energy); for most proteins studied so far, inactivation experiments were carried out at only one or two different temperatures (2–5). The activation energy (466.5 kJ/mol) for the inactivation of polyphenol oxidase in toluene with 8 mL/L water was reported (6), but the authors did not study the effect of other water concentrations or the use of other solvents. In relation to the behaviour of α-amylase in organic solvents, the only work found was reported by Blakeney and Stone (7), who used a mixture of ethanol–aqueous buffer and not an enzyme suspension in a dried organic solvent and did not present data on the thermal stability of the protein.
The development of enzyme-based TTI would therefore benefit from a better understanding of the effect of organic solvents and water content on the thermal stability of proteins in such unnatural environments, which is also becoming an interesting topic of fundamental research. This interest is mainly due to the potential of biocatalysis in dried organic solvents to obtain products whose synthesis is not easily accomplished in water and the possibility of performing fundamental studies such as protein dynamics and flexibility in an environment completely different from the traditional aqueous solution (8–10).
The main objective of this work is a systematic study of the effect of three organic solvents with different hydrophobicity on the stability of *B. amyloliquefaciens* α-amylase previously equilibrated at low water contents (1.5, 4.9 and 23.9 g of water per 100 g of dry solid). This would allow evaluation of the possibility of using organic solvents to change the thermal inactivation characteristics of this enzyme. From this the necessary kinetic parameters could be obtained to develop a TTI for monitoring thermal process lethality in the sterilization range of temperatures. Dodecane, octane and 1-octanol were chosen (with log*P* values of 6.6, 4.5 and 2.9, respectively). The log*P* parameter of a given

*To whom correspondence should be addressed.

solvent is the logarithm of the partition coefficient of that solvent between water and octanol and is a measure of its hydrophobicity. Solvents with higher values of log*P* are more hydrophobic (less hydrophilic)(11). A spin-off of the results could also be of interest to the field of biocatalysis, as previously mentioned.

Results were analysed using both the Arrhenius model (more common in chemical kinetics) and the Bigelow model (more usual in thermal processing of foods).

Materials and Methods

Lyophilization, equilibration, thermal treatment and activity analysis

The characteristics of the α-amylase used in this work (from *B. amyloliquefaciens*, later referred to as α-amylase), the lyophilization and thermal treatment conditions, the equilibration procedure above saturated salt solutions to obtain the desired water content and the activity analysis procedure are described in another work (12). The enzyme used was from a different lot to the enzyme used in the work cited. The procedures needed to carry out this work were modified as follows. After the thermal treatment and prior to activity analysis, 0.4 mL of Tris-HCl buffer pH 7.2 were added to the enzyme suspension and the mixture vigorously mixed to dissolve the enzyme. For the activity analysis, aliquots of the aqueous phase were then carefully withdrawn to avoid contamination of the organic phase.

Organic solvent handling and addition

Three hundred and fifty microlitres of commercially available dehydrated organic solvents (dodecane, octane and 1-octanol — Aldrich Chemical Company, Inc., Gillingham, Dorset, England) was added to the enzyme powder on the 7th day of equilibration. The vials containing the enzyme suspension were hand crimped tightly for 5 s, subjected to ultrasound for 2 min to form a fine suspension and kept at 4°C until the thermal inactivation was carried out. The organic solvents used were removed from the bottles according to the handling instructions provided by the manufacturer. This step was done very carefully to avoid atmospheric contact. The amount of water present in the organic solvents was found to be below the detection limit of the Karl Fischer titration. As the real amount of water bound to the enzyme was not determined, the water content of the solid matrix prior to the thermal treatment (obtained by equilibration over saturated salt solutions) was used to distinguish between the different water contents. For this work, the enzyme was equilibrated above saturated salt solutions of 0.11, 0.43 and 0.88 water activities, resulting in water contents of, respectively, 1.5, 4.9 and 23.9 g of water per 100 g of dry solid (13).

Data analysis

A thorough description of the model equations, regression procedures and statistical interpretation of the results is provided in a previous work (12).

Using the Bigelow model, the remaining activity of an enzyme inactivating according to a first order decay at constant temperature *T*, is given by:

$$\mathrm{Log}A = \mathrm{Log}\,A_0 - \left(\frac{t}{D_r \times 10^{\frac{T_r - T}{z}}}\right) \qquad \text{Eqn [1]}$$

while for the Arrhenius model one gets:

$$\mathrm{Ln}A = \mathrm{Ln}A_0 \times \exp\left(-\frac{E_a}{R \times T}\right) \qquad \text{Eqn [2]}$$

where A = total residual activity of the enzyme (ΔOD/min), A_o = total initial activity of the enzyme (ΔOD/min), t = time (min), 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, k_o = rate constant at an infinite reference temperature (min^{-1}), E_a = activation energy (J/mol) and R = gas constant (8.314 J/(mol.K)). A set of isothermal experiments, each at a different temperature, can be fitted to either equation with nonlinear regression, yielding the two kinetic parameters. The reference temperature used for the Bigelow model was 140°C (413 K).

Results and Discussion

Preliminary experiments

Preliminary experiments indicating linearity between enzyme concentration and initial activity (ΔOD/min), the stability of amylase during lyophilization and equilibration above the saturated salt solutions were previously reported (12). A similar method was used in this work. The effect of the organic solvents on α-amylase activity (with 1.5 and 23.9 g of water per 100 g of dry solid) was studied for up to 6 d of contact at 4°C and no significant change of activity was found (maximum of 3% loss of activity).

No activity regeneration was found for partially inactivated samples after storage at 4°C for 24 h and therefore only irreversible inactivation was studied.

It should be pointed out that the water concentration in each case is much smaller than that used in other works and also that water can be distributed between the enzyme and the organic solvent and therefore the concentration indicated relates to the total amount of water in both phases.

Inactivation data

It was found that an initial drop in activity occurred in all cases, that is, the first order behaviour assumed was

found for the second experimental point onwards, only. This is shown in **Figs 1** and **2** that present typical inactivation curves as a semi-log graph. This initial drop had already been found for inactivation without organic solvents (12). For mathematical interpretation of the results, it is possible to simply discard the initial value. It is however considered that a better understanding of this phenomenon would be valuable, particularly if such a system is going to be used as a TTI with predictive purposes. Horseradish peroxidase in similar conditions as those used in this work showed biphasic inactivation behaviour (13,14). This type of kinetics has been characterized using the two-fraction model (15). This model supposes the existence of two native enzyme populations (isoenzymes), one heat-labile and the other heat-stable, each inactivating by first order kinetics. In the present case of α-amylase the initial drop occurs very fast and is too small to allow for its measurement at the temperatures studied and therefore it is difficult to propose a two-fraction model with the data available.

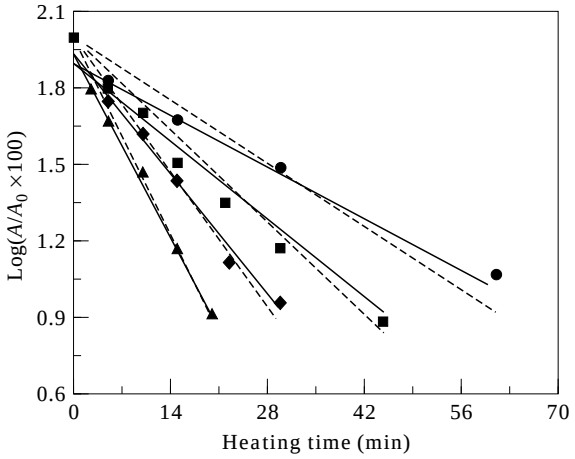

Fig. 1 Heat inactivation kinetics of α-amylase with 4.9 g of water per 100 g dry solid in dodecane at 135°C (●), 140°C (■), 145°C (◆), and 150°C (▲), and best fitted curve using one-step (– – –) analysis

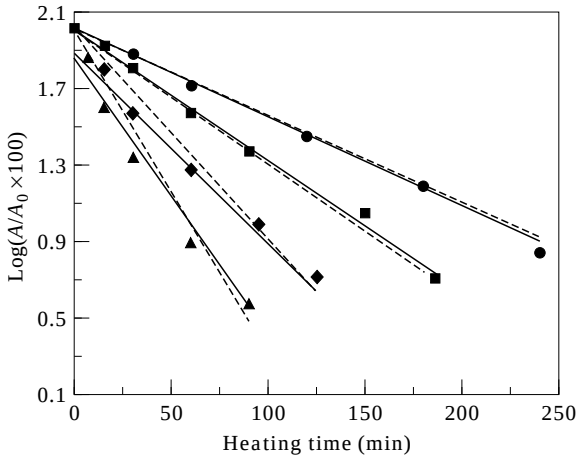

Fig. 2 Heat inactivation kinetics of α-amylase with 23.9 g of water per 100 g dry solid in 1-octanol at 125°C (●), 130°C (■), 135°C (◆), and 140°C (▲), and best fitted curve using one-step (– – –) analysis

Table 1 shows the kinetic parameters obtained with the Bigelow model and the Arrhenius model. The dried enzyme in hydrophobic organic solvents is much more stable than in aqueous buffer. D-values of 250 to 15 min were obtained in phosphate buffer at pH 7 for this enzyme at temperatures between 75 – 85°C. This is also noticeable in the inactivation temperatures (between 130 – 150°C for these conditions, compared to 80 –90°C in aqueous buffer solution).

The enzyme showed higher *z* values in these organic solvents than in aqueous Tris-HCl buffer solution at pH 8.5 (7°C) (16). The activation energies (or *z* values) are similar to those of α-amylase inactivation without solvents (12). This suggests that the inactivation mechanism was not changed by the introduction of the organic solvents. It has been reported that ribonuclease, chymotrypsin and lysozyme (at pH 8.0) inactivate in dried organic solvents due to protein aggregation (17). For glass-adsorbed α-chymotrypsin thermal inactivation, the high activation energy reported (6) also suggested a conformational change as the cause for inactivation. The low activation energy found for inactivation in both media (without and with organic solvents) for α-amylase indicates that a conformational

Table 1 Kinetic parameters obtained for inactivation of α-amylase from *Bacillus amyloliquefaciens* (means ± 95% confidence interval, for an average of 16 to 20 experimental points)

		One step method			
Solvent	Water content*	$D^{140°C}$ (min)	*z* (°C)	$1nK_o$	E_a (kJ/mol)
Dodecane	1.5	56.9±1.9	23.3±1.0	37.7±1.6	140.5± 5.4
	4.9	39.0±1.9	30.3±3.3	29.0±3.4	109.4±11.9
	23.9	54.5±1.1	25.6±1.7	34.9±2.6	130.9± 9.0
Octane	1.5	23.2±0.8	28.6±1.9	30.6±2.2	113.2± 7.6
	4.9	36.7±1.2	31.3±2.1	27.1±2.0	102.1± 6.8
	23.9	41.9±1.7	23.5±1.6	38.1±2.8	140.6± 9.7
1-octanol	1.5	28.9±1.3	23.5±1.9	37.5±3.2	137.4±11.1
	4.9	18.4±1.6	17.8±1.3	49.4±3.9	177.0±13.2
	23.9	143.4±6.4	26.1±2.1	32.9±3.0	127.2±10.4
Without solvents	1.5	41.0±4.4	24.5±2.7	n.d.	n.d.

*(g water per 100 g of dry solid).
n.d.= not determined.

change is not likely to play a significant role in the inactivation mechanism.

The significant stabilizing effect in the case of octanol at 23.9 g of water per 100 g dry solid is particularly interesting. This solvent has a less hydrophobic nature than the other two and also a higher dipolar moment, hydrogen bonding capacity and dielectric constant. Attempts have been made to relate the inactivation behaviour of enzymes in dried organic solvents with the organic solvents properties. A good correlation between the residual activity of glass-absorbed chymotrypsin heated for 60 min at 50°C and the log*P* parameter was found (2). It was reported that the best correlation for the enthalpy of unfolding of ribonuclease occurred with the dielectric constant (18). For example, it was reported that the addition of a small amount (70 mL/L) of 1-propanol (a hydrogen-bond former solvent) causes a remarkable decrease in the stability of α-chymotrypsin in dried octane (4). This effect can be interpreted in terms of disruption of hydrogen bonds in the protein, that contribute to its stability, and replacement by others with the polar organic solvent. It was reported that hydrogen-bond formers can partially replace water as activity activators for polyphenol oxidase and alcohol dehydrogenase (1). It was found that proteins in organic solvents have an increased hydrogen-bond network compared to aqueous solutions and consequently proposed that this is the cause of the experimentally observed properties of proteins in organic solvents (19). The experimental data reported in this work cannot answer the question of which properties of octanol are causing the increase in thermostability at the highest water content. However, and since both water content and the solvent may affect the thermal resistance of proteins, correlation of the inactivation characteristics with solvents' characterising parameters must take into account the exact amount of water present in the protein.

To evaluate the effect of higher water concentrations on inactivation, an experiment was carried out using octanol and α-amylase equilibrated at 0.43 water activity, but with a water concentration of 0.5 mL of water in 100 mL of octanol, about 100 times more water than the enzyme just equilibrated at 0.43 water activity. The D-value was 109 min at 110°C. This indicates a significant destabilising effect of water on thermal resistance. An even more pronounced effect of water on thermoinactivation was found for dried peroxidase when the water concentration in the organic solvent was increased 100 times (D-values falling from about 110 min at 130°C to about 80 min at 100°C) (13). This indicates that dried α-amylase is less sensitive to water content than peroxidase. This observation correlates well with the conclusion taken for the inactivation of solid-phase α-amylase without organic solvents (12).

The enzyme showed similar kinetic parameters for the three organic solvents, except for octanol with a water content of 23.9 g of water per 100 g of dry solid, which were also similar to those obtained in aqueous solvents at similar water contents (12). Thermal inactivation with aqueous solvents with a water content of 1.5 g of water per 100 g of dry solid was repeated with the same enzyme batch as that used for the inactivation experiments with the organic solvents (**Table 1**). The results indicated that the D-values were almost equal to those obtained for dodecane.

Statistical analysis

For an adequate statistical discussion on the influence of the organic solvent and water content on the inactivation kinetics, it is necessary to identify the joint confidence regions. In the present situation it was found that in all cases, the extreme values of the 90% joint confidence regions were close to the extremes of the individual 95% confidence intervals, as expected (12). **Figures 3** and **4** show the 90% joint confidence regions

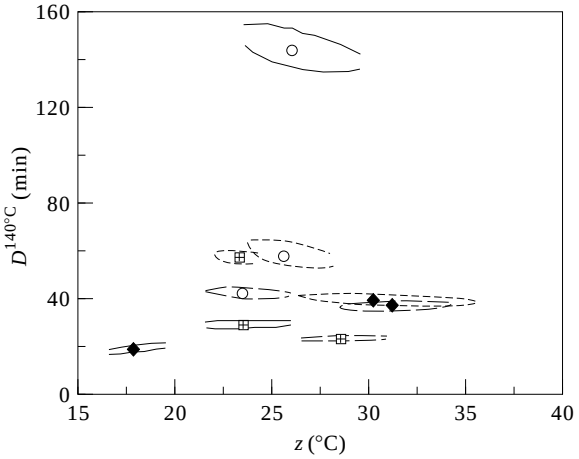

Fig. 3 90% joint confidence regions for dodecane (– – – – –), octane (— — —) and octanol (——) with water contents of 1.5 (⊞) 4.9 (◆) and 23.9 (○) g of water per 100 g dry solid for the Bigelow model (the symbols indicate the estimated solution using the one-step analysis)

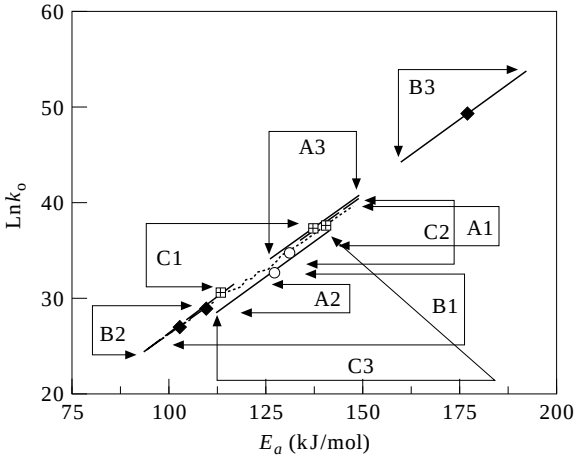

Fig. 4 90% joint confidence regions for dodecane (....., 1), octane (— — —, 2) and octanol (——), 3) with water contents of 1.5 (⊞, A) 4.9 (◆, B) and 23.9 (○, C) g of water per 100 g dry solid for the Arrhenius model (the limits for each water content and organic solvents are indicated in the graph)

for the results obtained with the Bigelow and Arrhenius models, respectively.

There is only one case where there is an overlap of two joint confidence regions (for dodecane with water content of 4.9 and 23.9 g of water per 100 g per dry solid). However, the parameters were generally similar. Only two situations were clearly different from the others, both for 1-octanol: with water content of 4.9 g of water per 100 g of dry solid a significantly lower *z*-value was obtained (less than 20°C while all other cases showed *z*-values around 25 – 27°C); and with water content of 23.9g of water per 100 g of dry solid a much higher D-value was found (around 140 – 150 min, while all others exhibited D-values around 20 – 60 min). It is interesting to compare the results to those reported for inactivation in aqueous solution at similar water content (12), because this will give the effect of the organic solvent addition. The D-value at 140°C was much higher for octanol with water content of 23.9 g of water per 100 g of dry solid, slightly higher for dodecane with water content of 1.5 and 23.9 g of water per 100 g of dry solid, lower for octanol with water content of 1.5 and 4.9 g of water per 100 g of dry solid and similar for all other cases. To compare the overall effect on stability it is necessary to take into account the *z*-value as well, but this was around 25 – 27°C in all cases, except for the one mentioned above, and therefore the effect on the D-value at the reference temperature is a good measure of the impact on stability.

Considering the results obtained with the Arrhenius model, the use of an infinite reference temperature leads to a highly collinear region. The error regions can be well approximated by straight lines. It is interesting to note that the slope of the joint confidence region is very similar in all cases and very close to the one for inactivation in aqueous solutions at similar water contents (12). This is due to the Arrhenius pre-exponential factor (k_o) being a reaction rate for an infinite temperature. The error regions differ basically: in the intercept, where it is noticeable that the result for 1-octanol with 23.9 g of water per 100g of dry solid is different (lower intercept); and in terms of the initial and final points of the region, where the most significant difference occurred for 1-octanol with 4.9 g of water per 100 g of dry solid. Bigelow's model showed similar results.

Overall, it can be concluded that octanol caused the most significant differences in the kinetic parameters, also providing the environment where the enzyme was most sensitive to water content.

The *z*-value of α-amylase thermal inactivation in the organic solvents studied is still higher than 10°C (the target for lethality assessment in low acid foods). The results obtained for α-amylase with octanol and 0.5 mL of water in 100 mL octanol indicate that solvent engineering, together with high water content, is a very promising way of obtaining different kinetic parameters in relation to the D-value only. To decrease the *z*-value other tools must be tried. On the other hand, the enzyme showed a comparable stability when inactivated without or with organic solvents. This indicates that α-amylase can withstand high temperatures in hydrophobic organic solvents, a property that could be advantageous when using this type of enzymatic system for biocatalysis.

Conclusion

Inactivation of solid-phase α-amylase from *B. amyloliquefaciens* in hydrophobic organic solvents showed first order inactivation kinetics. The enzyme is much more stable than in aqueous solutions and only slightly less stable than when inactivated in a solid matrix with the same water content but without organic solvents. The most different environments, compared to inactivation in aqueous solution, occurred with 1-octanol with a water content of 23.9 g of water per 100 g of dry solid (where the D-values were higher than those for inactivation without solvents and the activation energy was comparable) and with a water content of 4.9 g of water per 100 g of dry solid (with a significantly lower *z*-value, around 17 –19°C). In general, organic solvents and water content can be used to adjust the reaction rate, but not the activation energy.

Acknowledgements

The authors would like to acknowledge the Commission of the European Communities, AAIR programme (AIR1-CT92-0746), for financial support.

Author Jorge Saraiva acknowledges support from the Portuguese Junta Nacional de Investigação Científica e Tecnológica (JNICT).

References

1 Zaks, A. and Klibanov, A.M. The effect of water on enzyme action in organic media. *Journal of Biological Chemistry*, **263,** 8017–8021 (1988)

2 Reslow, M., Adlercreutz, P. and Mattiasson, B. Organic solvents for bioorganic synthesis. 1. Optimization of parameters for a chymotrypsin catalyzed process. *Applied Microbial Biotechnology*, **26,** 1–8 (1987)

3 Zaks, A. and Klibanov, A.M. Enzyme catalysis in organic media at 100°C. *Science*, **224,** 1249–1251 (1984)

4 Mozhaev, V.V., Poltevsky, K.G., Slepnev, V.I., Badun, G.A. and Levashov, A.V. Homogeneous solutions of hydrophilic enzymes in nonpolar organic solvents. New systems for fundamental studies and biocatalytic transformations. *FEBS Letters*, **292,** 159–161 (1991)

5 Chen, S.-T. and Wang, K.-T. Peptide synthesis in organic solvents catalyzed by an industrial alkaline protease 'Alcalase'. *Journal of the Chinese Chemical Society*, **39,** 683–691 (1992)

6 Estrada, P., Baroto, W., Castillon, M.P., Acebal, C. and Arche, R. Temperature effects on polyphenol oxidase activity in organic solvents with low water content. *Journal of Chemical Technology and Biotechnology*, **56,** 59–65 (1993)

7 Blakeney, A.B. and Stone, B.A. Activity and action pattern of *Bacillus licheniformis* α-amylase in aqueous ethanol. *FEBS Letters*, **186,** 229–232 (1985)

8 KHMELNITSKY, Y. L., LEVASHOV, A. V., KLYA, K. AND MARTINEK, K. Engineering biocatalytic systems in organic solvents with low water content. *Enzyme and Microbial Technology,* **10,** 710 (1988)
9 KLIBANOV, A. M. Enzymatic catalysis in anhydrous organic solvents. *Trends in Biological Sciences*, **14,** 141–144 (1989)
10 DORDICK, J. S. Enzymatic catalysis in monophasic organic solvents. *Enzyme and Microbial Technology*, **11,** 194 (1989)
11 LAANE, C., BOEREN, S., VOS, K. AND VEEGER, C. Rules for optimization of biocatalysis in organic solvents. *Biotechnology and Bioengineering,* **XXX,** 81–87 (1987)
12 SARAIVA, J., OLIVEIRA, J., HENDRICKX, M., OLIVEIRA, F. AND TOBBACK, P. Analysis of the inactivation kinetics of freeze dried α-amylase from *Bacillus amyloliquefaciens* at different moisture contents. *Lebensmittel-Wissenschaft und-Technologie,* **29,** 260–266 (1996)
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 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)
15 LING, A. C. AND LUND, D. B. Determinating kinetic parameters for thermal inactivation of heat resistant and heat labile isozymes from thermal destruction curves. *Journal of Food Science*, **43,** 1307–1310 (1978)
16 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)
17 VOLKIN, D. B., STAUBLI, A., LANGER, R. AND KLIBANOV, A. M. Enzyme thermoinactivation in anhydrous organic solvents. *Biotechnology and Bioengineering,* **37,** 843–853 (1991)
18 BATTISTEL, E. AND BIANCHI, D. Influence of the solvent properties on protein in organic media. *Proceedings of the Symposium Stability and Stabilisation of Enzymes.* Maastricht, The Netherlands, Elsevier Science Publishers B.V., pp. 13–20 (1993)
19 HARTSOUGH, D. S. AND MERZ, M., Jr. Protein Dynamics and solvation in aqueous and nonaqueous environments. *Journal of the American Chemical Society,* **115,** 6529–6537 (1993)