CHANGING THE THERMOSTABILITY OF *BACILLUS LICHENIFORMIS* α -AMYLASE

De Cordt, S., Saraiva, J.[‡], Hendrickx, M., Maesmans, G. and Tobback, P.

All authors are affiliated with the Center of Food Science and Technology, Unit Food Preservation, Faculty of Agricultural Sciences, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, België

[‡] Author J. Saraiva is affiliated with Escola Superior de Biotecnologia, Centro Regional da Universidade Católica do Porto, R. Dr. António Bernardino de Almeida, 4200 Porto, Portugal

[‡] The author acknowledges Junta Nacional de Investigação Científica e Tecnológica (JNICT) - Portugal for financial support

Abstract

We applied "solvent engineering" (i.e. variation of environmental conditions) to and/or immobilized *Bacillus licheniformis* α -amylase covalently onto porous glass beads. In this way, important alterations in its thermostability characteristics (k_{inact} , E_{Ainact}) were achieved.

1. INTRODUCTION

In the field of food and pharmaceutical preservation technology, there is a need for cheap, quick and, above all, reliable process evaluation, i.e. measurement of the "impact" of an applied heat treatment for pasteurization or sterilization. This is because on the one hand, the heat process must be lethal enough for pathogenic and/or spoilage micro-organisms in order to obtain a commercially safe product with extended shelf life, but on the other hand, it should be limited to what is strictly necessary since heat is detrimental also to most quality factors, e.g. (for foods) vitamin content, color intensity, texture. In other words, there is a problem of optimization.

Time-temperature integrators (TTI's)²⁻⁷ are devices that monitor the evolution (generally a decrease in concentration) of a product quality- or safety-parameter under the influence of a heat treatment. Concretely, these devices allow calculation of the concentration of this quality- or safety-parameter remaining after the applied heat process (C_t) from its concentration before heating (C_0), and the response levels of the TTI (i.e. activities in the case of an enzymic TTI) before and after the (same) heat treatment (X_0 and X_t , respectively) in a very simple way, and without needing to know the time-temperature profile of the applied heating process. This is because, at least on condition that the activation energy of the (e.g. destruction) process acting on the monitored parameter ($E_{\text{A,par}}$) be equal to the activation energy of the reaction that acts on the TTI ($E_{\text{A,TTI}}$), the next relationship holds, whatever the profile of temperature vs. time:

$$\ln(C_0/C_t)/k_{\text{ref,par}} = \ln(X_0/X_t)/k_{\text{ref,TTI}} \quad \text{for reaction orders } (n) = 1 \quad (1)$$

and

$$(1/k_{\text{ref,par}}) * (C_t^{1-n} - C_0^{1-n}) / (n-1) = (1/k_{\text{ref,TTI}}) * (X_t^{1-n} - X_0^{1-n}) / (n-1) \quad \text{for reaction orders } (n) \neq 1 \quad (2),$$

where $k_{\text{ref,par}}$ and $k_{\text{ref,TTI}}$ are the rate constants of the reactions occurring to the monitored parameter and to the TTI, respectively, at a chosen reference temperature. If the TTI is an enzyme, this reaction is its irreversible thermal inactivation.

Stabilization and, more generally, alteration of an enzyme's thermostability characteristics ($k_{\text{inact,ref}}$ and E_{Ainact}), are of interest in this field because of two reasons.

The first one is implied in the condition that $E_{\text{A,par}} = E_{\text{A,TTI}}$, which must be fulfilled for eqs. 1 and 2 to be valid and hence, for an enzyme to be able to function as a TTI. Most often the E_{Ainact} of an enzyme that is a good candidate to become a TTI (i.e. that meets other requirements of an economical and practical kind), is not equal to $E_{\text{A,par}}$ and hence must be changed. Second, the temperature range of interest in pasteurization and sterilization is from about 60 to 120° C and hence the k_{TTI} ($=k_{\text{inact}}$ for an enzymic TTI) must be sufficiently low in this zone in order to make X_t a detectable value. In other words: there should not be an immediate complete inactivation of the enzymic TTI in the temperature range of interest. From here it is almost evident that many "candidates" for becoming a TTI must be stabilized first i.e. their inactivation rate constants must decrease.

To achieve these goals, we applied "solvent engineering", i.e. variation of the concentrations of cosolvents or solutes that influence k_{inact} and/or E_{Ainact} and covalent immobilization.

2. MATERIALS AND METHODS

2.1. Materials

The α -amylase used was *Bacillus licheniformis* α -amylase, purchased from Sigma Chemical Co. (type XII-A) as an aqueous solution containing 32 mg of protein/ml (Biuret) and 15 % NaCl. The specific activity of this preparation was 680 Sigma units/mg of protein.

The carrier material for the immobilized enzyme was silane coated CPC-Silica Carrier (Fluka) of 30-45 mesh and 375 Å pore size. Before use, the beads were sieved to (partly) remove particles with diameter < 0.4 mm.

2.2. Immobilization procedure

The enzyme was covalently bound onto glass beads using the procedure as described by Weetall⁸. 1 gram of glass beads was activated in 2.5 ml glutaraldehyde solution (25 % w/w in water) and 22.5 ml of Na_2HPO_4 - NaH_2PO_4 buffer (pH 7; 0.1 M) on an end-over-end rotator for 2 hours, at room temperature. After washing the activated beads with distilled water, 1 ml of enzyme solution (**) (dilution of stock solution in Na_2HPO_4 - NaH_2PO_4 buffer, pH 8, 0.1 M) was added, and the mixture was shaken horizontally for 4 hours, at room temperature. After reaction the unbound enzyme fraction was removed by washing with distilled water.

2.3. Thermal treatment

The thermal treatment was performed in a temperature controlled oil bath. During the heat treatment, enzyme samples, dissolved or suspended (if immobilized) in a studied medium, were contained in sealed capillary tubes or in Chrompack crimp top vials. The samples were cooled in ice immediately after withdrawal from the oil bath.

2.4. Activity measurement

The activity of α -amylase was measured spectrophotometrically at room temperature according to a procedure based on the progressive hydrolysis of the α -1,4 glucosidic bonds in p-nitro-phenyl- α -D-maltoheptaoside, thus releasing gradually p-nitrophenol, which absorbs maximally at 405 nm (Sigma Diagnostics). Activities were expressed in terms of the change in optical density per minute, calculated by linear regression of absorption vs. time.

It should be stressed that, as a consequence of the way in which the thermal treatment and activity measurement are executed, we follow a process of irreversible inactivation¹.

2.5. Data analysis

First-order rate constants (k_{inact}) (time units⁻¹) were determined by linear regression of $\ln(A)$ vs. heating time:

$$\ln(A_t) = \ln(A_0) - k_{\text{inact}} * t \quad (3)$$

In many instances, immobilized enzyme shows "biphasic" inactivation kinetics, i.e. k_{inact} decreases with increasing reaction time (apparent reaction order > 1). Then, the plot of $\ln(A)$ vs. time could be adequately approached by two intersecting straight lines, and from the slopes of these we determined a $k_{\text{l,inact}}$ and $k_{\text{s,inact}}$ (rate constants for "labile" and "stable" fraction, respectively). Activation energies were determined from Arrhenius plots.

3. RESULTS AND DISCUSSION

All the results are presented in the form of Arrhenius plots since they allow a clear view on two most important features: the relative orders of magnitude of k_{inact} -values (higher or lower position of lines with respect to each other) and differences in $E_{\text{A,inact}}$ (slopes of the lines).

3.1. Biphasic inactivation kinetics of immobilized enzyme

Before starting the following discussion, it is important to give some clarification about how we dealt with biphasic kinetics in this paper.

It is repeatedly observed that inactivation of immobilized enzymes proceeds through an initial fast phase, followed by a slower phase, whereas inactivation of the soluble counterpart can be adequately described as simple first order processes^{1,3-5,9-12}. This also holds true for *B.l.* α -amylase. There are several mathematical descriptions available to deal with such phenomena. For the purpose of data analysis in this paper, we chose to apply the following:

$$A = A_l * \exp[-k_{\text{l,inact}} * t] + A_s * \exp[-k_{\text{s,inact}} * t] \quad (4)$$

where A is the experimentally measured activity, A_s and A_l are the activities of the thermostable and thermolabile protein fractions, respectively, at time 0, and $k_{\text{s,inact}}$ and $k_{\text{l,inact}}$ their respective inactivation rate constants. This model assumes a mechanism of parallel first-order inactivation of the two protein fractions.

In this paper, for the biphasic cases, only $k_{\text{s,inact}}$ and $E_{\text{A,s,inact}}$ are reported.

3.2. Results without use of polyols or carbohydrates

The results of varying pH, Ca^{2+} and enzyme concentration, immobilization and loading density were extensively discussed in De Cordt *et al.*¹.

(*fig. 1*) For the dissolved enzyme, we observed simple first-order inactivation kinetics in all instances. While k_{inact} is very sensitive to environmental changes, E_{Ainact} fluctuations are negligible. To shift E_{Ainact} , more thorough changes (e.g. immobilization) are necessary. Extrinsic Ca^{2+} confers extra stability to the enzyme. However, its effect on k_{inact} is small compared to that of, e.g., pH. It appeared from our results that the optimum pH for high stability of *B.l. α-amylase* is at about 8.5. As to the influence of the initial enzyme concentration on thermostability, some apparently contradictory data are found in literature^{9,13-15}. From our findings, it appears that the thermostability of dissolved enzyme was favoured by high initial enzyme concentration.

(*fig. 2*) Immobilization brought about two remarkable consequences. First, the E_{Ainact} of enzyme, immobilized covalently on glass beads and suspended in a Tris/HCl buffer, was about half the E_{Ainact} of the enzyme, dissolved in the same buffer. Consequently, if one is comparing thermostabilities in terms of k_{inact} values, temperature ranges must be specified because the Arrhenius plots of dissolved and immobilized enzyme intersect and beyond the intersection point, the relative order of stability reverses. Second, the immobilized enzyme showed biphasic inactivation kinetics in some studied cases. Only the results of the most stable fraction are shown then.

For the rest, alike the situation with dissolved enzyme, the variation of E_{Ainact} upon changing pH, $[\text{Ca}^{2+}]$ or enzyme loading densities is negligible, while k_{inact} is however very sensitive to such environmental parameters. Especially increasing the enzyme loading density on the carrier showed to be an effective means for stabilization of the immobilized α -amylase.

3.3. Results with use of polyols or carbohydrates

(*figures 3 and 4*) Addition of particular solutes has since very long been used for improving the storage stability of proteins, but several of these additives can evenso increase a protein's resistance to thermal inactivation. As to this subject, a lot of apparently conflicting literature data exist, and some authors think that generalizations in this field are seriously hampered because variation in hydrophilicity/hydrophobicity among proteins¹⁶, particularly in their surface patterns (i.e. the number and distribution of nonpolar, polar and charged groups)^{17,18} can lead to contrary effects of one and the same additive on different proteins. However, polyols of three or more carbons or carbohydrates generally stabilize proteins. Such effects are mostly discussed in terms of "preferential protein hydration", and other features that probably are mutually related, namely: changes in the protein's chemical potential^{19,20}, changes in water activity of the solvent system²¹, degree of water organization²², hydrogen-bond rupturing capacity of water molecules²³, strengthening of hydrophobic bonds in the protein²⁴, changes in the solvent dielectric constant²⁵, and "structure-making" or "structure-breaking" properties of solutes on the solvent²⁶.

If, however, the solute in question is a product- or substrate-analogue, a cofactor or any other substance for which there (is) (are) (a) binding site(s) on the protein, a stabilizing effect can be readily explained by the specific binding of this solute to native protein^{25,27}.

As to our results, all tested polyols (glycerol, mannitol, sorbitol) and carbohydrates (starch and sucrose) showed to be powerful stabilizers, at least so in specified temperature ranges. That is, for both dissolved and immobilized

Fig.1: comparison of thermostability of enzyme dissolved in Tris/HCl buffer, where * represents the situation where [enz.] = 1/125, pH = 6.9, $[Ca^{2+}] = 25\text{ppm}$, □ where [enz.] = 1/125, pH = 8.5, $[Ca^{2+}] = 50\text{ppm}$, ◇ where [enz.] = 1/50, pH = 8.5, $[Ca^{2+}] = 125\text{ppm}$, Δ where [enz.] = 1/100, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$, and for contrast: # represents immobilized enzyme, loading density = 1/100, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$

Fig.2: comparison of thermostability of immobilized enzyme submerged in Tris/HCl buffer, where * represents the situation where the enz. loading density = 1/100, pH = 8.5, no Ca^{2+} added, □ where enz. loading density = 1/100, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$, ◇ where enz. loading density = 1/100, pH = 8.5, $[Ca^{2+}] = 140\text{ppm}$, Δ where enz. loading density = 1/50, pH = 7.5, $[Ca^{2+}] = 70\text{ppm}$, # where enz. loading density = 1/50, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$, ○ where enz. loading density = 1/1000, pH = 8.5, $[Ca^{2+}] = 50\text{ppm}$, □ where enz. loading density = 1/1000, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$, + where enz. loading density = 1/250, pH = 8.5, $[Ca^{2+}] = 70\text{ppm}$, and for contrast: * represents dissolved enzyme, [enzyme] = 1/125, pH = 8.5, $[Ca^{2+}] = 50\text{ppm}$

In this paper, enzyme loading densities on the glass beads are expressed in terms of the dilution of the enzyme solution that was added to the activated beads in the immobilization procedure (see (**)) in Section 2.2). For dissolved enzyme, concentrations are given in a similar way: "1/x" signifies an x-fold dilution of the *B.l.* α -amylase solution described in Section 2.1.

Fig.3: comparison of thermostability of dissolved enzyme, where * represents the situation where [enz.] = 1/600 and [starch] = 10%(w/w) in Tris/HCl buffer^(*), □ where [enz.] = 1/600 and [starch] = 40%(w/w) in Tris/HCl buffer^(*), ◇ where [enz.] = 1/600 and [glycerol] = 50%(v/v) in Tris/HCl buffer^(*), △ where [enz.] = 1/600 and [mannitol] = 30%(w/w) in Tris/HCl buffer^(*), # where [enz.] = 1/600 and [sucrose] = 30%(w/w) in Tris/HCl buffer^(*), * where [enz.] = 1/10 and [sorbitol] = 60%(w/w) in Tris/HCl buffer^(*), and for contrast: + represents the situation where no polyols or carbohydrates were added to the Tris/HCl buffer^(*) ([enz] = 1/100)
^(*):specifications of the used Tris/HCl buffer: 0.05 M,pH = 8.5,[Ca²⁺] = 70ppm

Fig.4: comparison of thermostability of immobilized enzyme (loading density 1/50), where * represents the situation where [glycerol] = 50%(v/v) in Tris/HCl buffer^(*), □ where [mannitol] = 30%(g/g) in Tris/HCl buffer^(*), ◇ where [starch] = 40%(g/g) in Tris/HCl buffer^(*),and for contrast: # represents the situation where no polyols or carbohydrates were added to the Tris/HCl buffer^(*)

^(*):specifications of the used Tris/HCl buffer: 0.05 M,pH = 8.5,[Ca²⁺] = 70ppm

In this paper, enzyme loading densities on the glass beads are expressed in terms of the dilution of the enzyme solution that was added to the activated beads in the immobilization procedure (see (**) in Section 2.2). For dissolved enzyme, concentrations are given in a similar way: "1/x" signifies an x-fold dilution of the *B.l.* α -amylase solution described in Section 2.1.

enzyme the $E_{A_{inact}}$ is even so changed compared to the corresponding enzyme system without polyol or carbohydrate added, so that the $\ln(k_{inact})$ vs. $(1/T)$ lines intersect. For dissolved enzyme, glycerol (50% v/v) and mannitol (30% w/w) cause the $E_{A_{inact}}$ to almost reduce by 50% and sucrose (30% w/w) even by 75%. For immobilized enzyme, in all studied cases (glycerol 50% v/v; mannitol 30% w/w; starch 40% w/w) the $E_{A_{inact}}$ is about doubled compared to the case without polyol or carbohydrate. Hence it appears that high concentrations of polyols and carbohydrates, as well as immobilization, can alter the reaction mechanism.

Concerning the type of inactivation kinetics, it must be noted that, alike the cases without polyol or carbohydrate, we observed a simple first order decay for dissolved enzyme in all circumstances, while for immobilized enzyme it showed now distinctly biphasic, then less clearly or even not at all biphasic, depending on the applied temperature and environmental conditions.

4. CONCLUSIONS

The optimal pH for thermostability of *B.l.* α -amylase is at about 8.5. Higher Ca^{2+} and enzyme concentration lead to better thermostability, i.e. lower k_{inact} values, but $E_{A_{inact}}$ is unaffected by variations in either one or more of the above environmental parameters. It is, however, reduced by 50% upon covalent immobilization. Increasing the loading density of enzyme on the carrier can raise the thermostability. Mannitol, glycerol, sorbitol, sucrose and starch can also increase the protein's resistance to thermal inactivation, at least in specified temperature ranges because, alike immobilization, they can cause drastic changes in $E_{A_{inact}}$. Whereas for dissolved enzyme we observed simple first-order activity decay in all the studied cases, immobilized enzyme showed now distinctly biphasic kinetics, then less clearly or even not at all, depending on temperature and environmental conditions.

5. REFERENCES

1. DE CORDT, S., VANHOOF, K., HU, J., MAESMANS, G., HENDRICKX, M. AND TOBBACK, P. Thermostability of soluble and immobilized α -amylase from *Bacillus licheniformis*. *Biotechnol. Bioeng.* **40** (1992), 396-402.
2. TAOUKIS, P.S. AND LABUZA, T.P. Applicability of time-temperature indicators as shelf life monitors of food products. *Journal of Food Science* **54** (No.4) (1989), 783-788.
3. WENG, Z. A time-temperature-integrator for thermal processing of foods: A case study on immobilized peroxidase. Doctoral dissertation nr.201 (1991), Faculty of Agricultural Sciences, Katholieke Universiteit Leuven.
4. WENG, Z., HENDRICKX, M., MAESMANS, G. AND TOBBACK, P. Immobilized peroxidase: a potential bioindicator for evaluation of thermal processes. *Journal of Food Science* **56** (No.2) (1991), 567-570.
5. DE CORDT, S., HENDRICKX, M., MAESMANS, G. AND TOBBACK, P. Immobilized α -amylase from *Bacillus licheniformis*: a potential enzymic time-temperature integrator for thermal processing. *International Journal of Food Science and Technology* (december 1992), in press.
6. HENDRICKX, M., WENG, Z., MAESMANS, G. AND TOBBACK, P. Validation of a time-temperature-integrator for thermal processing of foods under pasteurization conditions. *International Journal of Food Science and Technology* **27** (1992), 21-31.

7. TOBBACK, P., HENDRICKX, M., WENG, Z., MAESMANS, G. AND DE CORDT, S. The use of immobilized enzymes as TTI-systems in thermal processing. In: "Advances in Food Engineering" (1992), 561-575. Ed. R.P. Singh and M.A. Wirakartakusumah. CRC press Inc., London.
8. WEETALL, H.H. Covalent coupling methods for inorganic support materials. In: "Methods in Enzymology: Immobilized enzymes" 44 (1976), 134-148. Ed. K. Mosbach. Academic Press, New York.
9. ULBRICH, R., SCHELLENBERGER, A., DAMERAU, W. Studies on the thermal inactivation of immobilized enzymes. *Biotechnol. Bioeng.* 28 (1986), 511-522.
10. KESTNER, A.I. Immobilized enzymes. *Russian Chemical Reviews* 43 (No.8) (1974), 690-706.
11. SCHELLENBERGER, A., ULBRICH, R. Protein stabilization by blocking the native unfolding nucleus. *Biomed. Biochim. Acta* 48 (No.1) (1989), 63-67.
12. MONSAN, P. AND COMBES, D. Effect of water activity on enzyme action and stability. In: "Enzyme Engineering 7." *Annals N.Y.Ac.Sci.* New York 434 (1984), 48-60. Eds. Laskin A.I., Tsao G.T. and Wingard L.B.Jr.
13. TOMAZIC, S.J., KLIBANOV, A.M. Mechanisms of irreversible thermal inactivation of *Bacillus* α -amylases. *J. Biol. Chem.* 263 (No.7) (1988a), 3086-3091.
14. TOMAZIC, S.J., KLIBANOV, A.M. Why is one *Bacillus* α -amylase more resistant against irreversible thermoinactivation than another? *J. Biol. Chem.* 263 (No.7) (1988b), 3092-3096.
15. VIOLET, M., MEUNIER, J.-C. Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α -amylase. *Biochem. J.* 263 (1989), 665-670.
16. COMBES, D., YOOVIDHYA, T., GIRBAL, E., WILLEMOT, R.-M. AND MONSAN, P. Mechanism of enzyme stabilization. In: "Enzyme Engineering 8." *Annals N.Y.Ac.Sci.* New York 501 (1987), 59-62. Eds. Laskin A.I., Mosbach K., Thomas D. and Wingard L.B.Jr.
17. FINK, A.L. Effects of cryoprotectants on enzyme structure. *Cryobiology* 23 (1986), 28-37.
18. GRAY, C.J. Additives and enzyme stability. *Biocatalysis* 1 (1988), 187-196.
19. ARAKAWA, T. AND TIMASHEFF, S.N. Stabilization of protein structure by sugars. *Biochemistry* 21 (1982), 6536-6544.
20. TIMASHEFF, S.N. AND ARAKAWA, T. Stabilization of protein structure by solvents. In: "Protein structure: a practical approach", (1989), 331-344. Ed. Creighton, T.E. IRL Press at Oxford University Press.
21. COMBES, D. AND MONSAN, P. Effect of polyhydric alcohols on invertase stabilization. In: "Enzyme Engineering 7". *Annals N.Y.Ac.Sci.* New York 434 (1984), 61-63. Eds. Laskin A.I., Tsao G.T. and Wingard L.B.Jr.
22. MONSAN, P. AND COMBES, D. Enzyme stabilization by immobilization. *Methods in Enzymology* 137 (1988), 584-598.
23. GERLSMA, S.Y. Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives. *The Journal of Biological Chemistry* 243 (No.5) (1968), 957-961.
24. GERLSMA, S.Y. The effects of polyhydric and monohydric alcohols on the heat induced reversible denaturation of chymotrypsinogen A. *Eur.J.Biochem.* 14 (1970), 150-153.
25. MORIYAMA, S., MATSUNO, R. AND KAMIKUBO, T. Influence of dielectric constants and ligand binding on thermostability of glucoamylase. *Agric.Biol.Chem.* 41 (No.10) (1977), 1985-1993.
26. BACK, J.F., OAKENFULL, D. AND SMITH, M.B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* 18 (No.23) (1979), 5191-5196.
27. PACE, C.N. AND MCGRATH, T. Substrate stabilization of lysozyme to thermal and guanidine hydrochloride denaturation. *The Journal of Biological Chemistry* 255 (No.9) (1980), 3862-3865.