Influence of microwave radiation on free *Candida antarctica* lipase B activity and stability

Barbara Réjasse,* Thierry BessonEn, Marie-Dominique Legoy and Sylvain Lamare

Received 18th July 2006, Accepted 23rd August 2006
First published as an Advance Article on the web 4th September 2006
DOI: 10.1039/b610265d

The influence of microwave heating on free *Candida antarctica* lipase B activity and stability was studied over the temperature range from 40 to 110 °C. Concerning the lipase activity, identical initial rate and conversion yield were obtained under microwave radiation and classical thermal heating for the alcoholysis between ethyl butyrate and butanol in a solvent-free system. On the other hand, the kinetics of the free lipase inactivation in butanol appears to be influenced by the heating mode. The Arrhenius plot obtained under classical heating was linear over all the temperature range studied whereas a biphasic Arrhenius plot was obtained under microwaves. The non-classical effect of the microwave heating on the initial rate of the enzymatic inactivation was thus dependent on the temperature of incubation.

Introduction

Microwave radiation as an energy source to heat is today widely used in organic chemistry. Indeed, an electromagnetic field of high frequency (2.45 GHz) induces molecular rotation of dipolar species, which is accompanied by intermolecular friction and subsequent dissipation of energy by heating in the core. Reduction in reaction times, enhancement in conversions and sometimes in selectivity have been reported concerning mainly solvent-free reactions conducted under a microwave field.[1a–c] It is not very clear whether these rate enhancements are purely due to thermal effects caused by the heating rate or whether some non-thermal effects, resulting from the uniqueness of the microwave dielectric heating mechanisms, are involved.[2,3]

In enzymatic synthesis, the use of microwave radiation remains limited. Literature on this subject is still poor and often controversial. A few studies deal with microwave-assisted enzymatic *trans*-esterifications carried out in non-aqueous media. Some of these report initial rate,[4–6] conversion yield[7,8] and enantioselectivity[9,10] enhancements when the reaction medium was heated at a specified temperature by microwaves rather than by conventional heating. The molecular mechanisms for these results are seldom discussed and are not well understood. Until now, the influence of the microwave radiation on the enzymatic stability has not been investigated and has not been taken into account.

We recently reported the influence of microwave heating on the activity and stability of immobilized *Candida antarctica* lipase B at 100 °C in a non-aqueous medium.[11] The results showed that in our conditions, the lipase activity was not influenced by the heating mode whereas enzyme inactivation could be slowed down by using microwave dielectric heating. In the present work, we further investigated the microwave effect on *Candida antarctica* lipase B properties. In these experiments, lipase was used in a free form. The latter is more thermosensitive than the immobilized form and enabled us to vary the temperature of the study from 40 to 110 °C. The temperature influence on the enzymatic activity and stability, according to the heating mode, has been studied. Lipase activity was tested with a *trans*-esterification model reaction between ethyl butyrate and butanol (alcoholysis) in a solvent-free medium (Scheme 1). Lipase stability was studied in storage conditions in butanol, with a thermodynamic water activity (a_w) close to 0, considering that microwave effects on enzymatic stability have been found in these conditions with the immobilized form of the lipase.[11]

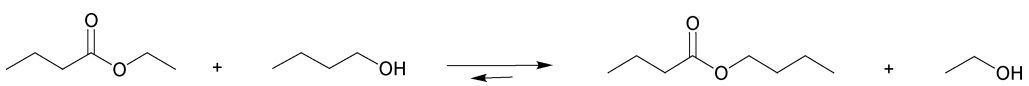
Laboratoire de Biotechnologies et de Chimie Bioorganique, CNRS FRE-2766, UFR Sciences Fondamentales et Sciences pour l'Ingénieur, Bâtiment Marie Curie, Université de la Rochelle, F-17042, La Rochelle cedex 1, France. E-mail: barbararejasse@yahoo.fr

Results and discussion

Alcoholysis activity of the free lipase

The alcoholysis activity of the biocatalyst for the model reaction was determined at 100 °C and $a_w \approx 0$, by adding different amounts of the free lipase to a mixture of substrates (ethyl butyrate–butanol, 15 : 44 mmol). Experiments were carried out under conventional and microwave heating to detect a possible effect of the heating mode on the enzymatic activity.

The alcoholysis reaction, which did not occur in the absence of the enzyme, produces butyl butyrate and ethanol and is reversible. Its initial rate was determined by following the appearance of butyl butyrate in the first fifteen minutes of reaction. Initial rates, obtained according to the quantity of lipase and to the heating mode, are shown in Fig. 1.



Scheme 1 The model reaction.

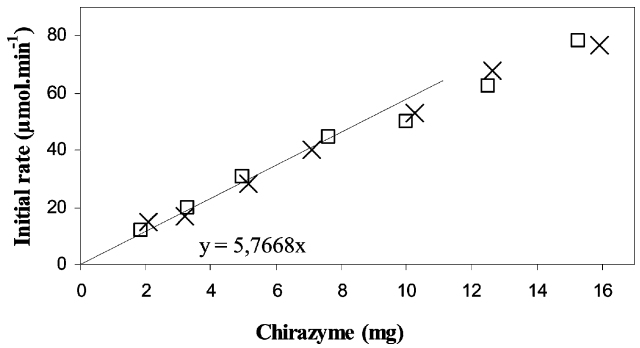


Fig. 1 Determination of the alcoholysis activity of the free lipase at 100 °C and $a_w \approx 0$ (□, conventional heating; X, microwave heating).

The enzymatic activity of the free *Candida antarctica* lipase B is similar under both heating modes. The same result was obtained with the immobilized form of the lipase.[11] In the present conditions, the free biocatalyst has an alcoholysis activity of 5.8 µmol min^{-1} mg^{-1}.

The initial rate of alcoholysis increases linearly with lipase quantity up to 8 mg of Chirazyme. The study of enzymatic stability is carried out with 5 mg of free lipase and the corresponding profile of reaction at 100 °C is shown in Fig. 2.

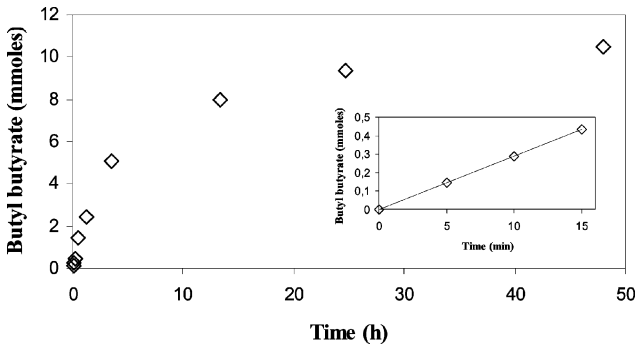


Fig. 2 Kinetics of the alcoholysis reaction with 5 mg of Chirazyme, at 100 °C and $a_w \approx 0$.

The kinetics of the alcoholysis reaction were similar under conventional and microwave heating. With 5 mg of Chirazyme, the reaction was in initial rate conditions in the first fifteen minutes whereas equilibrium of the reaction was reached after 48 h. The quantity of butyl butyrate synthesized was about 11 mmol, which corresponds to 70% of the initial ethyl butyrate quantity. Traces of butyric acid resulting from the hydrolysis of ethyl- and butyl butyrate have been detected. However, the hydrolysis reaction was not significant and can be ignored under both heating modes.

Temperature effect on the alcoholysis enzymatic activity

The influence of temperature on enzymatic activity was studied by varying the temperature of the reaction, from 40 °C to 110 °C. The alcoholysis initial rate was independent of the heating mode over all the temperature range tested (Fig. 3). A maximum activity level of about 10 µmol min^{-1} mg^{-1} appears at 60 °C. Increasing the temperature further to 60 °C resulted in a spectacular drop in enzymatic activity, showing the thermal inactivation of the enzyme.

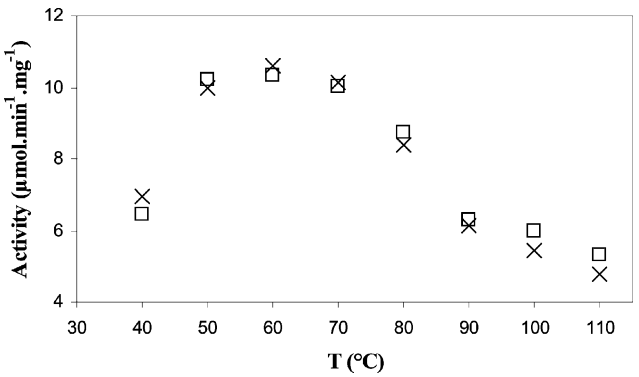


Fig. 3 Influence of temperature on alcoholysis enzymatic activity (□, conventional heating ; X, microwave heating).

Enzymatic stability in storage conditions in butanol

The stability of the free *Candida antarctica* lipase B was studied in storage in butanol under both heating modes, from 70 °C to 110 °C. The thermal irreversible deactivation curves obtained are shown in Fig. 4.

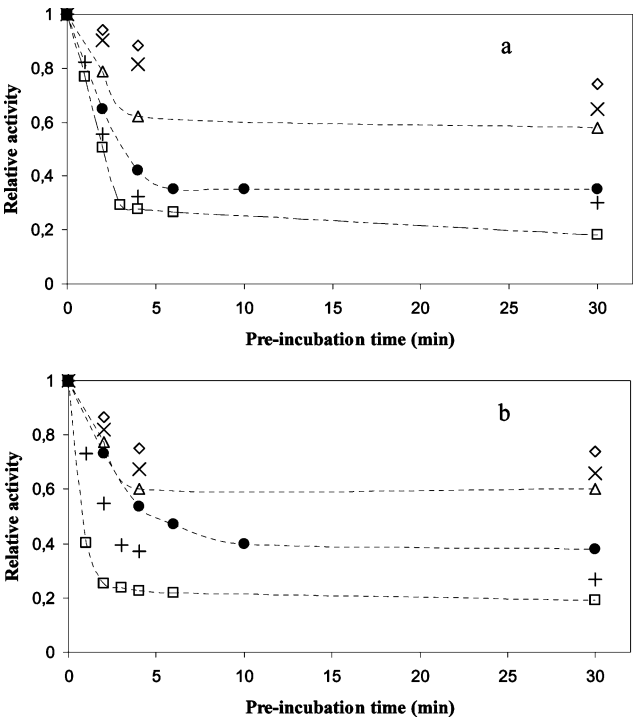


Fig. 4 Inactivation kinetics of *Candida antarctica* free lipase in butanol at 70 °C (◇), 80 °C (X), 90 °C (△), 100 °C (●), 105 °C (+) and 110 °C (□), under conventional (a) and microwave (b) heating.

Over all the temperature range tested, residual enzymatic activities decrease rapidly with time, in the first minutes of incubation. Even faster inactivation kinetics are observed when higher values of temperature are adopted. In all cases, the enzymatic inactivation process can be considered as completed from 30 min of incubation, since the loss of enzymatic activity was extremely slow thereafter.

The remaining enzymatic activity, after 30 min of incubation, was identical for both heating types (Fig. 5). For temperatures of incubation lower than 90 °C, the free lipase was relatively

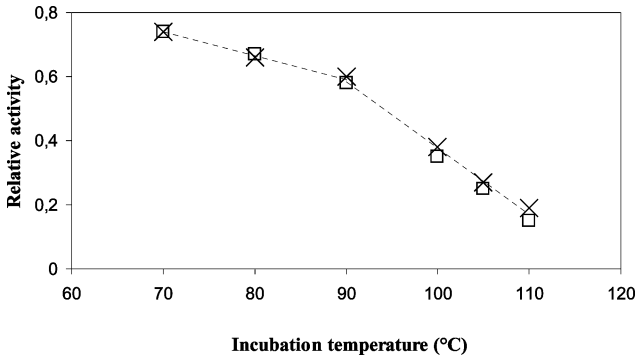


Fig. 5 Residual activity of *Candida antarctica* free lipase after 30 min of incubation in butanol, under conventional (□) and microwave (X) heating.

stable, since less than 40% of its activity was lost. From 90 °C, the inactivating effect of butanol was clearly enhanced with increasing temperature, which reflects the cooperativity of the inactivation process.

The initial rate of the enzymatic inactivation was determined in the first 4 minutes of incubation, according to the incubation temperature and to the heating mode. The rate deactivation constant (k_d) at a specified temperature has been calculated from the gradient of the respective pseudo-first order plot of $\ln(E/E_0 \times 10)$ *versus* pre-incubation time (Fig. 6), where E is the residual enzymatic activity obtained after pre-incubation and E_0 is the enzymatic activity obtained without pre-incubation. Different k_d values could be obtained at a given temperature, depending on the heating mode used for the enzymatic pre-incubation.

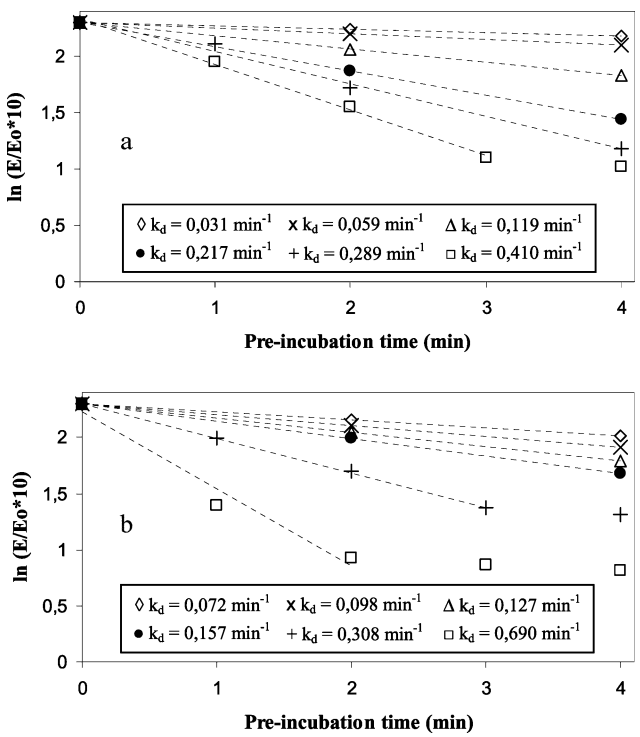


Fig. 6 Determination of deactivation constant (k_d) of *Candida antarctica* free lipase in butanol at 70 °C (◊), 80 °C (X), 90 °C (△), 100 °C (●), 105 °C (+) and 110 °C (□), under conventional (a) and microwave (b) heating.

The temperature dependence of the first order inactivation rate constant can be expressed by the Arrhenius relationship as indicated in eqn (1):

$$\ln k_d = -E_a/(RT) + \ln A \quad (1)$$

where E_a is the activation energy, A, a constant and R the gas constant (8314 J mol^{-1} K^{-1}). The relation between $\ln k_d$ and $1/T$ obtained according to the heating mode is presented in Fig. 7.

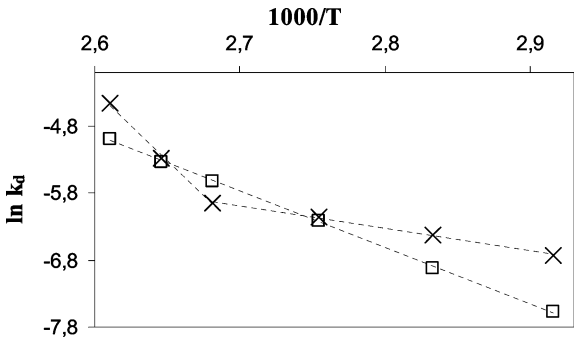


Fig. 7 Arrhenius plots for deactivation of *Candida antarctica* free lipase in butanol (□, conventional heating; X, microwave heating).

Under conventional heating, the Arrhenius plot was linear over all the temperature range tested. This enabled us to determine the activation energy (E_a) of the inactivation process. E_a was found to be equal to 70 kJ mol^{-1}. Under microwave heating, the temperature dependence of k_d was different. The Arrhenius plot indicates two inactivation regimes (two slopes) of the free lipase according to the temperature of incubation. At temperatures higher than 100 °C, the rate of enzyme inactivation was strongly affected by a temperature-dependent inactivation regime, *i.e.* the enzyme inactivation rapidly increased with increasing temperature. At lower temperatures, a slower inactivation regime was observed, again different from that obtained under classical heating. Thus, at 100 °C, free lipase inactivation in butanol was slowed down when the reaction medium was heated by microwaves rather than by conventional heating. The same result was obtained with the immobilized form of the lipase.[11] However, at other temperatures, the inactivation process can also be accelerated under microwave radiation, which denoted two different mechanisms of inactivation according to the heating mode.

Conclusions

Several researchers have envisaged the existence of a special effect of microwave radiation on enzymatic catalysis. In our conditions, microwave radiation does not have any effect on the activity of alcoholysis of *Candida antarctica* free lipase B, but seems to affect the enzymatic stability in storage conditions.

Although the same enzymatic state was observed after 30 min of incubation in butanol under microwave and conventional heating, the initial rate of the enzymatic inactivation was different depending on the heating type used during the incubation and the variation of rate measured was dependent on the incubation temperature.

The effect of the microwave radiation on the inactivation rate could be of non-thermal origin. Indeed, the interaction of the microwave field with the enzyme could induce a modification

of the local enzymatic configuration, as suggested in previous studies.[12] This modification would modify the stability. A specific organization of the reaction medium in the microwave field, leading to a change in the pre-exponential factor A[6] or in the activation energy in the Arrhenius equation,[13] can also be envisaged.

However, a thermal/kinetic origin of the effect of the microwave radiation cannot be excluded. The presence of "hot spots" has been reported in various experiments[3,14] and a heterogeneous repartition of the temperature in the incubation medium could modify the enzymatic inactivation rate when the medium was heated by microwaves rather than by conventional heating.

More research is required to explain the real effect of microwave radiation on the enzymatic stability that we have observed. *In situ* structural studies must be now carried out to determine if microwave and conventional heating are producing different structural enzymatic intermediates during the inactivation process, or if only the rate of the enzymatic transitions is influenced by the heating type.

Experimental

Enzymatic and chemical materials

Chirazyme® L-2 Lyo, a purified and lyophilized preparation of *Candida antarctica* lipase B, in a free form, was procured from Boehringer Mannheim GmbH (Germany). Ethyl butyrate, butyl butyrate, butyric acid were purchased from Sigma Chemical Co. (USA). Butanol, ethanol and acetonitrile were obtained from Carlo Erba Reagenti (Italy). All substrates and solvents were of the highest purity (99% minimum).

Biocatalyst was stored at 4 °C with P_2O_5 under vacuum and substrates were dried with $MgSO_4$ before use, to fix the thermodynamic activity of water of the experiments close to 0. This thermodynamic parameter is defined as follows:[15]

$$a_w = P_{p,w}/P_{p,w,ref} = \text{relative humidity}/100 \quad (2)$$

where $P_{p,w}$ is the vapor pressure of water in equilibrium with the system used and $P_{p,w,ref}$ is the vapor pressure at the same temperature above pure water.

Microwave equipment

Focused microwave irradiations were carried out at atmospheric pressure with a synthewave S402 Prolabo microwave reactor (300 W, monomode system, capacity of the quartz reactor used: 10 and 70 mL). The apparatus possesses a variable speed rotation, visual control, irradiation monitored by PC computer, infrared measurement and continuous feed temperature control (by PC).[16] The temperature of the reaction mixture was controlled using an algorithm, which allows the temperature to be set at a given value by varying the power between 10 and 60 W to operate under the electromagnetic field over the reaction. Although this model is not yet commercially available reactions can be reproduced at atmospheric pressure, in the same conditions, using the CEM Discover reactor which possesses similar characteristics.[17]

Gas chromatographic analysis

The GC analysis was performed with a Hewlett Packard model 5890A instrument equipped with flame ionization detector (FID) and an OV 01 fused silica capillary column (Chrompack, France). The split ratio was 68 : 1.2. Injector and detector were kept at 220 and 250 °C respectively. Carrier gas was nitrogen and the flow rate in the column was 1.2 ml min^{-1}. Hydrogen and air were supplied to the FID at 45 and 350 ml min^{-1} respectively.

For the separation of the substrates and products, diluted in acetonitrile, the column temperature was programmed to increase from 60 to 190 °C, at 15 °C min^{-1}.

Alcoholysis model reaction

A mixture of substrates, ethyl butyrate and butanol (15 : 44 mmol), was placed in a 10 mL quartz vessel and heated at the specified temperature in an oil bath (conventional thermal heating) or in the microwave oven. When the temperature of the mixture was stabilized, the reaction was started by the rapid addition of the free lipase. The temperature of the reaction medium was then continuously monitored. Periodically, 40 µl of the reactional medium were withdrawn and immediately centrifuged (1 min, 14000 rpm) in order to eliminate the enzyme and to stop the reaction. 10 µl of supernatant was then diluted in 400 µl acetonitrile and then analysed by GC.

All experiments were repeated three times to reduce measurement activity errors. The errors never exceeded 5%. The showed graphs are based on the average value of the three measurements.

Enzymatic stability in storage conditions in butanol

Butanol (44 mmol) was placed in the quartz vessel and heated to the specified temperature under conventional or microwave heating. As soon as thermal equilibrium was reached, 5 mg of Chirazyme were rapidly added to initiate the pre-incubation. The temperature was maintained constant (±1 °C) by microwave radiation or conventional heating for all the duration of the incubation period.

At the end of the pre-incubation time, ethyl butyrate (15 mmol) was added to the mixture to test the residual activity of the lipase. The initial rate of the enzymatic alcoholysis was calculated in the first fifteen minutes of the reaction and was compared to the initial rate obtained in identical conditions without enzyme pre-incubation.

References

1 For recent books on microwaves in chemistry: (*a*) *Microwaves in organic synthesis*, ed. A. Loupy, Wiley-VCH Verlag Gmbh & Co. KGaA, Weinhein, 2002; (*b*) B. L. Hayes, in *Microwave synthesis: chemistry at the speed of light*, CEM Publishing, Matthews, USA, 2002; (*c*) *Microwave-Assisted Organic Synthesis*, ed. P. Lidström and J. P. Tierney, Blackwell Publishing, Oxford, 2005.
2 L. Perreux and A. Loupy, *Tetrahedron*, 2001, **57**, 9199–9223.
3 A. de la Hoz, A. Diaz-Ortiz and A. Moreno, *Chem. Soc. Rev.*, 2005, **34**, 164–178.
4 M. C. Parker, T. Besson, S. Lamare and M. D. Legoy, *Tetrahedron Lett.*, 1996, **37**, 8383–8386.
5 I. Roy and N. Gupta, *Tetrahedron*, 2003, **59**, 5431–5436.

6 G. D. Yadav and P. S. Lathi, *Enzyme Microb. Technol.*, 2006, **38**, 814–820.
7 M. Gelo-Pujic, E. Guibé-Jampel, A. Loupy, S. A. Galema and D. Mathé, *J. Chem. Soc., Perkin Trans. 1*, 1996, 2777–2780.
8 G. D. Yadav and P. S. Lathi, *J. Mol. Catal. A: Chem.*, 2004, **223**, 51–56.
9 J. R. Carrillo-Munoz, D. Bouvet, E. Guibé-Jampel, A. Loupy and A. Petit, *J. Org. Chem.*, 1996, **61**, 7746–7749.
10 G. Lin and W. Y. Lin, *Tetrahedron Lett.*, 1998, **39**, 4333–4336.
11 B. Réjasse, S. Lamare, M. D. Legoy and T. Besson, *Org. Biomol. Chem.*, 2004, **2**, 1086–1089.
12 J. N. Rodriguez-Lopez, L. Fenoll, J. Tudella, C. Devece, D. Sanchez-Hernandez, E. de los Reyes and F. Garcia-Canovas, *J. Agric. Food Chem.*, 1999, **47**, 3028–3035.
13 W. Huang, Y. M. Xia, H. Gao, Y. J. Fang, Y. Wang and Y. Fang, *J. Mol. Catal. B: Enzym.*, 2005, **35**, 113–116.
14 C. O. Kappe, *Angew. Chem., Int. Ed.*, 2004, **43**, 6250–6284.
15 P. Halling, *Enzyme Microb. Technol.*, 1994, **16**, 178–206.
16 P. Jacquault, *French Pat.*, 1991, **9**, 116286; P. Jacquault, *Eur. Pat.*, 1992, 549495.
17 A complete description of this reactor was recently published: D. J. Ferguson, *Mol. Diversity*, 2003, **7**, 281–286.