COMMUNICATION TO THE EDITOR

Reactivity and Stability of Mycelium-Bound Carboxylesterase from *Aspergillus oryzae*

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Received 10 November 2000; accepted 1 September 2001

Abstract: The reactivity and thermostability of a novel mycelium-bound carboxylesterase from lyophilized cells of *Aspergillus oryzae* are explored in organic solvent. Ethanol acetylation was selected as reference esterification reaction. High carboxylesterase activity cells were used as biocatalyst in batch esterification tests at $12.5 < S_o < 125$ mmol L^{-1}, $5.0 < X_o < 30$ g L^{-1}, $0.49 < \log P < 4.5$ and $30 < T < 80$°C, as well as in residual activity tests after incubation at $40 < T < 90$°C. The starting rates of product formation were used to estimate with the Arrhenius model the apparent activation enthalpies of the enzymatic reaction (29–33 kJ mol^{-1}), the reversible unfolding (56–63 kJ mol^{-1}), and the irreversible denaturation (22 kJ mol^{-1}) of the biocatalyst. © 2002 John Wiley & Sons, Inc. *Biotechnol Bioeng* **77**: 232–237, 2002.

Keywords: carboxylesterase; *Aspergillus oryzae*; reactivity; stability; ethanol acetylation; kinetics; ethyl acetate

INTRODUCTION

The use of enzymes in organic media is a rapidly developing technology which promises to offer several advantages in industrial application, such as increased solubility of hydrophobic substrates, improved product and enzyme recovery, reduced risk of contamination, enhanced thermostability and altered specificity. The most important advantage is that hydrolases may catalyze kinetically or thermodynamically unfavored reactions, such as synthesis over hydrolysis (Koskinen and Klibanov, 1996). However, because the catalytic efficiency of enzymes in organic solvents is usually less than in aqueos media (Klibanov, 1997), several procedures were proposed to increase their activity (Carrea and Riva, 2000) and solubility (Sereti et al., 2001). Lipases, and more generally carboxylesterases, are among the enzymes of major interest for industrial application, being used in a number of chemo-, regio-, and stereoselective ester synthesis in organic solvents (Bornscheuer and Kazlauskas, 1999; Kamiya et al., 1999; Koskinen and Klibanov, 1996; Schmid et al., 1999).

Most of the reported applications are interesterification reactions, while direct condensation between alcohol and free acid is usually more difficult to obtain (Flores et al., 2000). Direct esterification is an equilibrium that leads to the formation of water, therefore it is unfavored by an increase in water activity in the organic medium (Svensson et al., 1994). Moreover, direct esterification is hampered not only by water formation but also by the strong inhibition exerted by the free acid on the lipase activity, especially when acetic acid is employed (Bourggarros et al., 1998; Claon and Akoh, 1993, 1994; De Castro et al., 1997; Langrand et al., 1990). Therefore, the discovery of new microbial lipases and esterases suited for this application is very attractive. Lyophilized mycelium of a newly isolated strain of *A. oryzae* showed an outstanding ability to promote direct acetylation and was employed to point out the factors influencing the production of cell-bound carboxylesterases (Molinari et al., 1996, 2000).

In view of future application, the reactivity and thermostability of this system are kinetically investigated. The acetylation of ethanol is taken as reference reaction because of a relative lack of information on this biotransformation (Langrand et al., 1990; Molinari et al., 2000) as well as the significance of the product as flavor and fragrance component.

MATERIALS AND METHODS

Aspergillus oryzae MIM, identified at Central Bureau voor Schimmelcultures, Baarn, Holland, was main-

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Contract grant sponsor: Italian National Council of Research (CNR), Biotechnology Project
Contract grant number: 97.01019, PF 115.08601

DOI 10.1002/bit.10124

tained on malt extract (8 g L^{-1}, agar 15 g L^{-1}, pH 5.5), cultured in 500-mL Erlenmeyer flasks containing 100 mL of medium and incubated for 48 h at 28°C on a reciprocal shaker (100 spm). The media, containing a basal medium (Difco yeast extract, 1 g L^{-1}; $(NH_4)_2SO_4$, 5 g L^{-1}; K_2HPO_4, 1 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1}; pH 5.8) or soytone (5 g L^{-1}), were supplemented with carbon sources selected according to the desired level of carboxylesterase activity. High-activity or low activity cells were prepared using Tween 80 or glucose plus olive oil, respectively. Suspensions of spores at different concentrations were used as inocula.

The biocatalyst was prepared submitting the mycelium to five successive vacuum filtrations alternated to four washings with 1:10,000 Tween 80 water solution, *n*-hexane, 0.1 *M* phosphate buffer at pH 7.0, and deionized water, respectively. The cells were then resuspended in deionized water, homogenized for 5 min (Silverson L2R, Silverson Machines LTD, Waterside, U.K.), frozen at −20°C, and finally lyophilized (Alfa Criosec, Milano, Italy) at a plate temperature of 25°C.

Ethyl acetate synthesis was studied in triplicate in 10 mL screw-capped test tubes by suspending lyophilized mycelium in the selected solvent (5 mL), adding ethanol and acetic acid up to the desired equimolar levels and magnetically stirring the reaction mixtures under the selected conditions. The initial rates were expressed as mmol of product formed in one hour per liter.

Ethanol and ethyl acetate concentrations were determined by gas chromatography as previously described (Molinari et al., 2000).

Kinetics of enzyme irreversible denaturation were studied at each selected temperature mixing the same amount of lyophilized *A. oryzae* cells corresponding to 20 g L^{-1} with 5 mL of *n*-heptane for variable times in 6 different test tubes. For every tested temperature (40, 50, 60, 70, 80, and 90°C), acetic acid and ethanol were daily added into different test tubes up to constant equimolar levels (50 and 100 mmol L^{-1}) in order to estimate the carboxylesterase residual activity. After heat treatment, the samples were rapidly cooled at room temperature to ensure efficient refolding of the enzyme molecules reversibly inactivated. The starting rates of ester formation obtained at 25°C with cells heated at the selected temperature for variable times (24, 48, 72, 96, and 120 h) were expressed as fraction of that obtained with cells not submitted to thermal treatment and used for estimation of the kinetic and thermodynamic parameters of the irreversible denaturation of the biocatalyst.

THEORY

Esterification Mechanism and Kinetics

Considering the reversible character of the reactions catalyzed by carboxylesterases, a typical ping-pong mechanism can reasonably be thought for alcohol acylation in organic solvent, which is opposite to that demonstrated by Jencks (1987) for esters hydrolysis in water. First the enzyme, E, reacts with the acid, A, forming a non-covalent enzyme–acid complex, which is then transformed into a tetrahedral intermediate by the attack of a serine hydroxyl group onto the acid carbonyl. This intermediate collapses releasing water and a stable acyl–enzyme intermediate, E_A. The following nucleophilic attack of the alcohol, B, results in the formation of a new tetrahedral enzyme-product complex, E_P, that deacylates to release the free enzyme and the ester, P.

At the start of the reaction, the concentrations of both substrates keep practically unvaried at their starting values (A_o and B_o), whereas those of both products, ester and water, can be neglected, so both first and last steps can be considered irreversible. Besides, assuming steady-state conditions for the enzyme forms (E_A, E_P, and E) and supposing that the inactive fraction of the enzyme is negligible at the start of the reaction, we obtain, under conditions of equimolar levels of both substrates as those investigated in this study ($S_o = A_o = B_o$):

$$v_o = \frac{k_{cat} S_o}{K_m + S_o}, \tag{1}$$

where v_o is the starting rate of the ester formation, $k_{cat} = k_3 E_{t0}$ a biocatalyst kinetic constant, and K_m an apparent Michaelis constant including the kinetic rate constants of the above steps.

Mechanism and Kinetics of Thermal Inactivation of the Biocatalyst

A classic model proposed by Volkin and Klibanov (1989) describes the overall phenomenon of thermal inactivation of enzymes by a two-step process. A preliminary reversible step of thermal unfolding would be responsible for the formation of a less active conformation of the enzyme, E_I, which would be subsequently subject to an irreversible step, leading to an aggregated stable (inactive) protein, E_D:

$$E \overset{K_I}{\leftrightarrow} E_I \overset{k_d}{\rightarrow} E_D, \tag{2}$$

where K_I is the equilibrium constant of partial enzyme unfolding and k_d is the first-order rate constant of irreversible thermal denaturation.

Despite the simplicity of this model, it seems to be of general validity and to hold true for different proteins (Baptista et al., 2000), including enzymes in organic media. It is likely that the thermal inactivation of our mycelium-bound carboxylesterase is the result of the solvent molecules interacting with the system "membrane-lipase," which reversibly produces a conformational change disturbing the active conformation of the enzyme.

If the latter irreversible stage is much slower than the former, E and E_I can be considered in equilibrium, thus the overall process can kinetically be described by first-order kinetics:

$$v_d = k_d E_I, \quad (3)$$

where E_I is the total concentration of the active enzyme, following a thermal treatment of duration t. These mechanisms could be confirmed by Fourier-transform infrared spectroscopy (Sereti et al., 2001; Vecchio et al., 1996, 1999), which may give additional information about the structure of the biocatalyst and the alteration of this property by the interaction with the solvent at different temperature values.

Defining the activity coefficient, ψ, as the ratio of the total active enzyme concentration at time t to that at the beginning of the thermal treatment ($\psi = E_t/E_{t0}$), the first-order denaturation constant, k_d, can be estimated at different temperatures from the slopes of the straight lines obtained by plotting the experimental data of ln ψ versus time (Mozhaev, 1993).

RESULTS AND DISCUSSION

As explained in detail in a previous work (Molinari et al., 2000), the use of Tween 80 strongly enhanced cell-bound lipolytic activity. A strong induction of the hydrolytic activity was generally obtained when it was used at 5.0 g L^{-1}, while weak mycelium-bound activity was observed at higher concentration. Using glucose as carbon source resulted in poorer hydrolytic activity. Tests of ethanol acetylation, used as control esterification reaction in this study, demonstrated that the medium composed by 5.0 g L^{-1} Tween 80 and basal medium furnished mycelium with the highest specific activity, confirming the trend observed in hydrolytic reactions. It is likely that such a concentration of Tween 80 was the optimum for the final macrostructure of the system "membrane-lipase", which carries the maximum number of active lid-opened lipase molecules. The positive effect of Tweens as main carbon sources for obtaining high mycelium-bound carboxylesterase activity has been recently observed also with *Rhizopus oryzae* (Gandolfi et al., 2001). Lyophilized mycelium from pellets obtained using a spore concentration of 1.6×10^4 spores mL^{-1} after 72 h showed the highest activity and was used for the successive kinetic studies.

To study the effect of the biocatalyst concentration on the kinetics of ethanol acetylation, a set of 4 batch tests was carried out with high activity cells in *n*-heptane, using 100 mmol L^{-1} starting substrate concentration at 50°C and different biocatalyst concentrations (namely, 5, 10, 20, and 30 g L^{-1}). The high average biotransformation yield on starting substrate (0.83 mol mol^{-1}) demonstrates that the lyophilization protocol followed in this work ensured a water activity close to the optimum. The starting rate of ethyl acetate formation linearly increased from 3.9 to 13.8 mmol L^{-1} h^{-1} with increasing the biocatalyst concentration, X_o, from 5 to 20 g L^{-1}, following first-order kinetics. At X_o = 30 g L^{-1}, this parameter kept practically constant (13.7 mmol L^{-1} h^{-1}), consistently with zeroth order kinetics, thus pointing out the substrate concentration instead of that of biocatalyst as the determining factor under these conditions.

The influence of solvent on the apparent kinetic parameters was also investigated to provide some information on both enzyme structure and action mechanism. For this purpose, additional tests were performed in different solvents with increasing log P (Laane et al., 1987; Halling, 1994), using high activity cells at T = 50°C, S_o = 100 mmol L^{-1}, and X_o = 20 g L^{-1}. The starting rates of product formation in tetrahydrofuran (log P = 0.49) and isopropyl ether (log P = 2.2) were only 6% and 54% of that in *n*-heptane (log P = 4.0), whereas the acetylation was about 15% faster in isooctane (log P = 4.5). The progressive increase in carboxylesterase activity with the solvent hydrophobicity could be the result of an increased thermostability. This situation, already observed by Volkin et al. (1991) for other systems, would be consistent with the supposed hydrophobic nature of this biocatalyst and its link to the membrane (Molinari et al., 1995, 1996, 1998). In addition, it would confirm the hypothesis that its inactivation could be due to the interaction between enzyme and solvent molecules.

Further tests were carried out in *n*-heptane, at 50°C and X_o = 20 g L^{-1}, with high-activity cells, varying the starting equimolar concentrations of both substrates (ethanol and acetic acid), in order to verify whether this biotransformation actually follows the mechanism described by the Michaelis–Menten-type equation (1) and to calculate the related kinetic parameters. The experimental data of product concentration versus time were used to calculate the initial rate of ethyl acetate formation at different S_o values. Plotting these results in Figure 1 according to Lineweaver–Burk, an apparent Michaelis constant, K_m, of 121.4 mmol L^{-1} and a k_{cat} value of 28.3 mmol L^{-1} h^{-1} were estimated with satisfactory correlation (r^2 = 0.982). Although an apparent Michaelis–Menten model is too simple to get certain information about the mechanism of this complex biocatalytic system, the very high K_m value estimated in this work could be ascribed to the presence of diffusion limitations of substrates and products through the media.

The final part of this work was addressed to the effect of temperature on both the reactivity and stability of *A. oryzae* mycelium-bound carboxylesterase. For this purpose, additional experiments were carried out in *n*-heptane at X_o = 20 g L^{-1} and S_o = 50 and 100 mmol L^{-1}, varying the temperature from 30 to 80°C with intervals of 5°C. Two lots of lyophilized cells with differ-

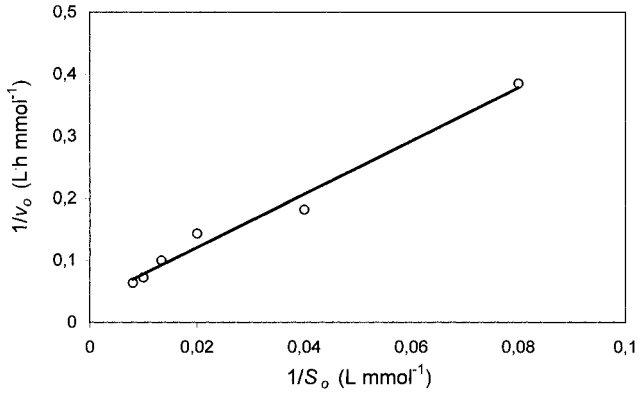

Figure 1. Lineweaver–Burk plot of ethanol acetylation by mycelium-bound carboxylesterase of *A. oryzae* in *n*-heptane. T = 50°C; X_0 = 20 g L^{-1}.

ent carboxylesterase activity were prepared using different carbon sources (either containing Tween 80 for high-activity cells or glucose for low-activity cells), in order to ascertain whether the observed activity variations can be ascribed to possible thermodynamic causes. To minimize the influence of activity loss due to irreversible denaturation of the biocatalyst, all kinetic results refer to the initial rates of ethyl acetate formation calculated within the first hour of each experiment.

Figure 2 shows this thermal effect in a semilog plot versus the reciprocal temperature. For both high-activity and low-activity cell preparations, below 55°C (side a of the figure) ln v_o decreased with increasing $1/T$, whereas it went in the opposite direction over that value (side b), with no appreciable influence of S_o. Such a high T threshold put in evidence the thermoresistance of the enzymatic system under consideration, which reminds one of that characterizing most lipases (Owusu et al., 1992). In addition, the positive effect of Tween 80 seems to indicate that the enzyme is a membrane-bound catalyst located in the external zone of the membrane.

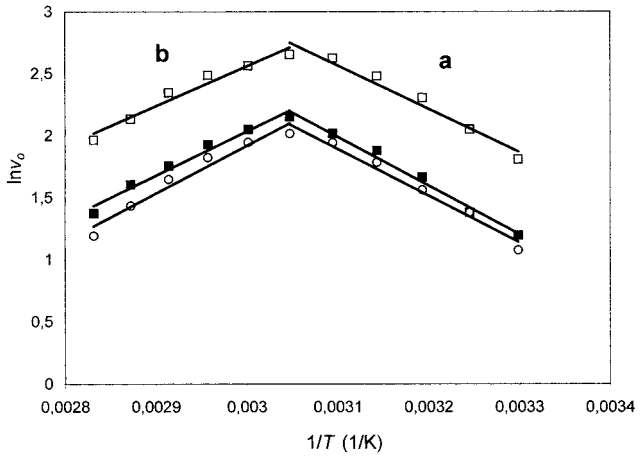

Figure 2. Arrhenius plots for the estimation of the thermodynamic parameters of both (a) ethanol acetylation by mycelium-bound carboxylesterase of *A. oryzae* and (b) reversible unfolding of the biocatalyst. Reference temperature: 50°C. Lowactivity cells grown on glucose plus olive oil, S_0 = 100 mmol L^{-1}: (■). Highactivity cells grown on Tween 80 plus olive oil: (□) S_0 = 100 mmol L^{-1}; (○) S_0 = 50 mmol L^{-1}.

Table I. Activation enthalpies (kJ mol^{-1}) estimated by the Arrhenius model for ethanol acetylation and reversible unfolding of mycelium-bound carboxylesterase from *A. oryzae* (reference temperature, 50°C).

	S_0 (mmol L^{-1})	Enzymatic reaction	Reversible unfolding
Low-activity cells	100	35.3	64.9
High-activity cells	100	31.8	58.5
	50	34.0	65.9

The activation enthalpies of both acetylation reaction and partial unfolding of the enzyme (Table I) were estimated with satisfactory correlation ($0.956 < r^2 < 0.987$) with the Arrhenius model from the plots of Figure 2. These values show only a little influence of the lipasic activity of the mycelium on the thermodynamic parameters and seem to support at qualitative level the above indication of external enzyme localization. A weak influence of starting substrate level on the thermodynamic parameters should also be noticed.

As early said, supposing that the latter first-order irreversible step of Eq. (2) is much slower that the former equilibrium, a kinetic study on denaturation can be performed assuming that also the overall enzyme activity decay can reasonably be described by first-order kinetics. The activity coefficient, ψ, was estimated as the ratio of the starting rate of product formation at S_o = 50 mmol L^{-1} with cells previously exposed at a given temperature for variable times to that obtained with cells not submitted to thermal treatment. The apparent first-order rate constant of enzyme denaturation, k_d, was then estimated at different temperatures with excellent correlation ($0.995 < r^2 < 0.999$) from the slopes of the straight lines in Figure 3. It should be stressed from the above results that mycelium-bound carboxylesterase followed the one-step decay typical of the classic first-

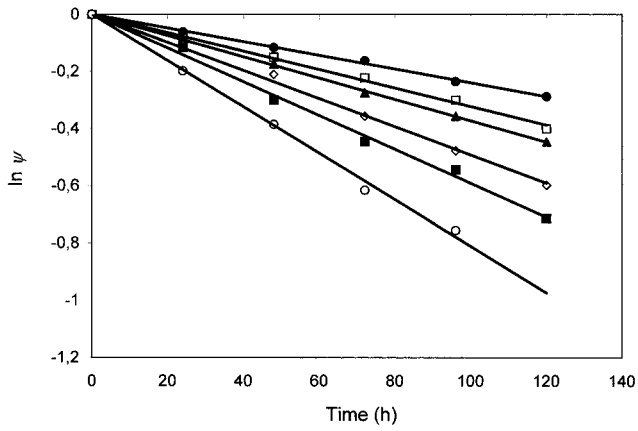

Figure 3. Semi-log plots of irreversible denaturation of mycelium-bound carboxylesterase of *A. oryzae* in *n*-heptane. T (°C): 40 (●); 50 (□); 60 (▲); 70 (◇); 80 (■); 90 (○).

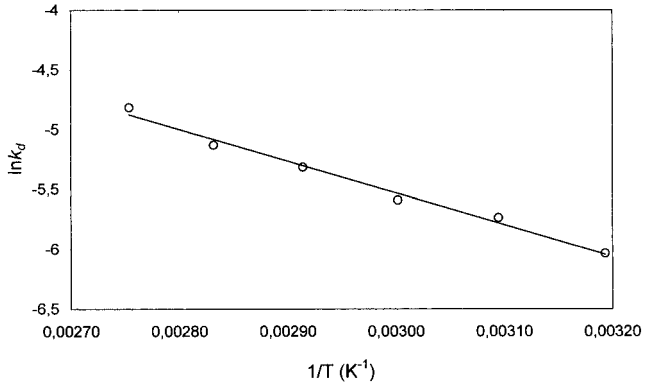

Figure 4. Arrhenius plot for the estimation of the thermodynamic parameters of irreversible denaturation of mycelium-bound carboxylesterase of *A. oryzae* in *n*-heptane.

order denaturation pattern, like that observed by Moreno et al. (1997) for both native and immobilized purified lipases, and that k_d progressively increased with temperature, according to the theory of the absolute reaction rate and to the Arrhenius equation. At 90°C, that is at the highest temperature tested in this work, only 0.8% of the starting enzyme activity was lost after one hour of heat treatment, which confirms the validity of the assumptions that (a) the irreversible denaturation is a much slower phenomenon than the reversible enzyme unfolding and (b) its negative contribution to the enzyme activity can be neglected at the start of each experiment. The temperature dependence of k_d is better evidenced in Figure 4, where the values of ln k_d are plotted versus $1/T$. The increased enzyme stability offered by its natural immobilization is also proved by comparison of the particularly high values of its activity half-life, $t_{1/2}$, at 40, 50, and 90°C (289, 215, and 85 h, respectively) with those calculated for native pure lipases (0.28–0.5 h at 50°C) or for commercial lipases immobilized onto different supports in aqueous medium (5–15 h at 50°C) (Moreno et al., 1997) or in organic solvents (3–16 h at 40°C) (Arcos et al., 2001).

The apparent activation parameters of irreversible denaturation ($\Delta E_d^{\#}$ = 24.8 kJ mol^{-1}; $\Delta H_d^{\#}$ = 22.1 kJ mol^{-1}; $\Delta G_d^{\#}$ = 116.8 kJ mol^{-1} at 50°C; $\Delta S_d^{\#}$ = −0.293 kJ mol^{-1} K^{-1}) were estimated by the Arrhenius model from the values of ln k_d (r^2 = 0.988). A comparative analysis of these thermodynamic parameters with those gathered in Table I and the peculiar monophasic activity loss evidenced in Figure 4 suggest a situation, for the system under consideration, which is completely different from that known for most lipases in aqueous media, thus confirming the significant role of the solvent in the mechanism of enzyme inactivation.

Despite of a lower activation enthalpy of irreversible denaturation, k_d of mycelium-bound carboxylesterase was in organic solvent 1–3 orders of magnitude lower than those of other lipases in aqueous medium (Arcos et al., 2001; Moreno et al., 1997; Owusu et al., 1992). This high thermoresistance is the result of a $\Delta S_d^{\#}$ value (−0.293 kJ mol^{-1} K^{-1}) which is much lower than those estimated by the above authors (varying from −0.092 to −0.186 kJ mol^{-1} K^{-1}).

Because of the complexity of the system and the use of simplified classic models, the present work should be considered as an attempt to explore this biocatalyst from the kinetic point of view rather than an absolute kinetic study and the results should be relative. Nevertheless, it is one of the few attempts made to separate the effects of reversible unfolding and irreversible denaturation of a biocatalyst on its activity (Baptista et al., 2000; Cioci, 1995).

This work was supported by the CNR Target Project on Biotechnology (no. 97.01019, PF 115.08601).

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