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Catalytic properties of *Candida antarctica* lipase B clusters solubilized in hexane

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Abstract

The objective of this work was to investigate the particle size and determine the catalytic competency of a solubilized lipase in hexane. Purified *Candida antarctica* lipase B (CALB) was solubilized in hexane using the non-ionic surfactant Span 60. The amount of surfactant was chosen so that complete coverage of the individual enzyme molecules with surfactant was not possible. Dynamic Light Scattering (DLS) was used to directly investigate the particle size of the solubilized entities. The enzyme was found to be solubilized in the form of clusters of lipase molecules with a radius of 37 ± 5 nm at 42°C, which we estimate to correspond to about 1200 CALB molecules. The solubilized enzyme clusters showed lower catalytic activity in a model esterification reaction in hexane compared with a commercial immobilizate of the same enzyme (Novozym 435). Further gains in catalytic activity may be possible by striving for true molecular-level dispersion of the enzyme in hexane.

Keywords: Candida antarctica, *solubilization, scattering, surfactant*

Introduction

Lipases are useful biocatalysts because of their ready availability combined with relatively low cost and versatility in organic synthesis (Decastro & Anderson 1995; Gandhi et al. 2000). The use of lipases in non-aqueous solvents, such as hexane, is attractive because they can transform substrates that are unstable or poorly soluble in water, to access novel reactivities and influence enantioselectivity via solvent engineering (Klibanov 2001). For example, at low thermodynamic water activity, esterification by lipases becomes thermodynamically favourable over hydrolysis. Undesirable side reactions that require water may be minimized in organic solvents and thermal stability may improve (Secundo & Carrea 2002).

Since enzymes are not soluble in organic solvents, they are often immobilized on solids such as polymer beads, porous glass or fumed silica (Liese et al. 2005; Würges et al. 2005). Another approach is to use surface-active substances, such as fatty acids or their derivatives, ionic surfactants to disperse the enzyme in an organic solvent (Basheer et al. 1995; Okahata et al. 1995), or aerosol OT or AOT (Wangikar et al. 1997). In the surfactant-based approach, one can target formation of reverse (water in organic solvent) micelles (Eicke 1980; Komives et al. 1994), where the enzyme resides in the aqueous phase. In the absence of free water, one can target solubilization of the enzyme molecules without a water phase by direct interaction of surfactant molecules with the enzyme.

This work is focused on enzyme solubilization in hexane. The number of surfactant molecules was deliberately reduced (i.e. surfactant-starved) and was estimated to be not physically sufficient to cover the surface of each enzyme molecule completely. Some authors (Paradkar & Dordick 1994) have reported apparently molecularly dispersed enzyme molecules solubilized in an organic solvent and in a

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surfactant-starved regime when surfactant-modified enzymes were extracted from water into solvents.

In this work, we solubilized purified free lipase B from *Candida antarctica* (CALB, E.C.3.1.1.3) in hexane using the non-ionic surfactant Span 60. The solubilization procedure was based on the literature (Basheer et al. 1995). The extent to which the solubilized CALB was agglomerated was analysed by dynamic light scattering (DLS), which can probe length scales in the order of 1–1000 nm (Murphy 1997).

Studies on surfactant-based solubilization, the physical result of solubilization and enzymatic activity of the preparations have been reported previously (Paradkar & Dordick 1994; Basheer et al. 1995; Okahata et al. 1995; Persson et al. 2002). The catalytic activity in solvents and the physical result of two different solubilization procedures for subtilisin Carlsberg and CALB using AOT were recently reported (Akbar et al. 2007). Akbar et al. do not comment on the relative amount of AOT available per enzyme molecule. One of the reported solubilization procedures was somewhat similar to this work, although the solubilized enzyme was dried in a gas stream, rather than lyophilized as reported here, and a biphasic system was used to bring enzyme and surfactant in contact as opposed to the monophasic aqueous/ethanol system used by us. A modified procedure by Akbar et al. eliminated entirely the step of first dissolving the enzyme in aqueous buffer before contacting with a solution of the surfactant in a solvent. No significant changes in enzyme secondary structure were found by Akbar et al. after introducing the solubilized preparations into solvent. Clusters of CALB with a diameter of about 34 ± 4 nm were found by dynamic light scattering after the modified solubilization procedure (no pre-dissolving of enzyme), while the solubilization more similar to our work resulted in entities of 7.5 ± 2.2 nm diameter. Our results indicating CALB clusters of 37 ± 5 nm radius corroborate these results reasonably well considering the differences in surfactant, the different solvent for surfactant dissolution, the ratio of surfactant to enzyme and freeze drying vs. evaporative drying.

The goal of this work was to determine the size of the solubilized CALB entities in hexane using dynamic light scattering and their catalytic activity using a model reaction. Size measurements with DLS indicated that the 'surfactant-starved' regime resulted in particles of a size consistent with solubilized enzyme molecule clusters, rather than individual molecules. The preparation showed lower catalytic activity compared with the commercial immobilized enzyme preparation Novozym 435 (Novozymes A/S, Bagsvaerd, Denmark).

Materials and reagents

10-mm UV glass cuvettes (3.0 mL, with PTFE cover), Pyrex glass tubes (Teflon-faced rubber-lined cap, 16×125 mm) and 4-mL screw capped glass vials (Fisher Scientific, Pittsburgh, PA) were used. Tris-buffer Sigma 7–9 (tris hydroxymethylaminomethane, 99% pure) was from Sigma Aldrich. Aqueous sodium hydroxide solution (5.0 N, Fisher grade), aqueous hydrochloric acid solution (6.0 N, Fisher grade), ethanol (denatured, HPLC grade), hexane (optima grade), methylene chloride (HPLC grade) and acetone (certified ACS.) were all from Fisher Scientific. Nitrogen (technical grade) was from Linweld, Manhattan, KS. Purified water was prepared by carbon adsorption and mixed bed ion exchange followed by reverse osmosis.

Span 60 (a sorbitan ester, Sigma Chemicals) was used as the surfactant. Span 60 is a mixture of approximately 50% stearic (C18) and 50% palmitic (C16) acid esters resulting in an average calculated molecular weight (MW) of 417 g/mol. The critical micelle concentration (CMC) of both compounds in hexane is 1.7×10^{-5} M (22°C). The head group cross-sectional area for stearic acid ester and palmitic acid ester molecules in hexane is, respectively, 38 and 37 Å^2 (Peltonen et al. 2001). An average cross-sectional area of 37.5 Å^2 is used here later to estimate the required area to adsorb one surfactant molecule perpendicular to a surface.

CALB has a molecular weight of 33 kDa (Uppenberg et al. 1994). X-ray structure analysis reveals a size of $30 \times 40 \times 50$ Å (Uppenberg et al. 1994). In contrast to many other lipases, CALB does not contain an amphiphilic peptide loop or 'lid' near the catalytic site (Martinelle et al. 1995).

A generous gift of 100 mg of chromatographically purified free (not immobilized) CALB (1700 LU/mL, hydrolysis of tributyrin in aq. solution, 30°C) was provided by Novozymes A/S, Bagsvaerd, Denmark. The lipase was received as a frozen solution in an aqueous buffer which was freeze-dried (10-MR-TR, VirTis Co., −45°C, *c.* 160 mtorr overnight, final drying at 25°C, storage in a closed vial at 8°C).

Experimental methods

The solubilization treatment was performed according to a procedure reported elsewhere (Basheer et al. 1995). 500 mL of 5 mM aqueous Tris buffer was adjusted to pH 5 with 0.12 N hydrochloric acid. Approximately 0.375 g Span 60 was dissolved in 10 mL ethanol by stirring and heating to 40°C. 13.2 mg of the purified and freeze-dried CALB was added to 40 mL of the Tris buffer solution at *c.* 4°C in a thermostated double-walled 75-mL glass

reaction vessel. The enzyme solution was vigorously stirred until it appeared clear. 0.8 mL of the Span 60/ethanol solution was added dropwise to the agitated solution. This turned the enzyme solution turbid and small particles (estimated at less than 1 mm) appeared, which was probably aggregated Span 60. The sample was transferred to a covered Erlenmeyer flask and sonicated for 15 min at room temperature. The sub-millimetre particles disappeared and the sample appeared homogeneously turbid. The solution was transferred to a clean and covered reaction vessel and was stirred for 2 h at 5°C. The sample was then transferred to glass test tubes (*c.* 5 mL) for centrifuging (15 min, 1380 ***g***, Fisher Scientific Centrific Model 228). The supernatant was decanted and no free Span 60 was observed in the decanted solution. This indicates that at least the vast majority of Span 60 was associated with the precipitate from centrifugation, along with the enzyme. The precipitate from centrifugation (here termed $CALB_{sol}$) was frozen at −20°C overnight and then freeze-dried for at least 24 h (final drying 25°C, 145 mtorr).

Figure 1 outlines the preparation procedure of the samples for dynamic light scattering measurements. 0.4–0.5 mg/mL of $CALB_{sol}$ was transferred to a 4-mL screw capped glass vial followed by the addition of 2 mL of water-saturated hexane. Water saturated hexane and water saturated Span 60/hexane were prepared for reference. All samples were heated to 40°C for *c.* 10 min and kept in a water bath to minimize temperature variations prior to DLS. Prior to any DLS measurements, all samples were filtered (0.45-μm syringe filter, Whatman, 13 mm, PTFE) into a preheated cuvette (Figure 1, step 2) and held at 40°C. Based on preliminary observations, we found that unfiltered solutions would turn cloudy with time whereas filtered samples would remain clear. DLS measurements were made at either room temperature (Figure 1, step 3b) or near 40°C (Figure 1, step 4).

In the DLS set up, a He–Ne laser (633 nm, 40 mW, Spectra Physics 125) was focused into the cell. The scattered light was collected by a lens that imaged the scattering volume onto a slit and to a FW 130 photomultiplier (angle between 10 and 30°). These pulses were amplified, discriminated, and correlated by a commercial correlator (ALV5000), and the resulting exponential correlation spectra were fitted to a first order decay:

$$C(t) = B + Ae^{-t/\tau_c}, \tag{1}$$

where $C(t)$ is the intensity autocorrelation function, B is the final y-axis value (signal), A is the starting y-axis level minus B, t is the time, and τ_c is the correlation time of the diffusing particle in the solution (Berne & Pecora 2000). It can be shown that the correlation time is given by:

$$\tau_c = \frac{1}{2Dq^2}, \tag{2}$$

where D denotes the translational diffusion coefficient and q is the scattered wave vector. The diffusion coefficient D can be calculated by the Stokes–Einstein equation:

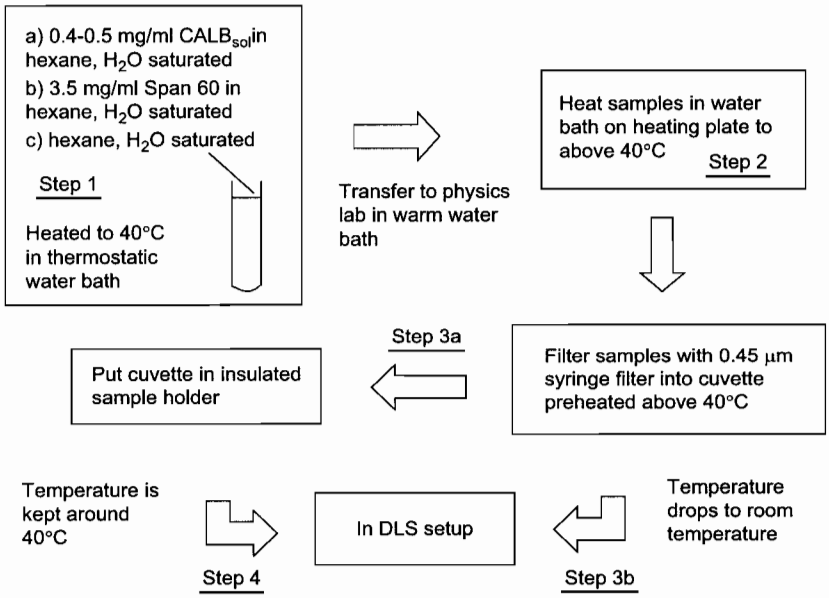

Figure 1. Procedure of the sample preparation for dynamic light scattering measurements.

$$D = \frac{k_B T}{6\pi\eta R_m}, \quad (3)$$

where k_B is the Boltzmann constant, $T(K)$ is the temperature, $\eta(T)$ is the solution viscosity and R_m is the mobility equivalent radius of a sphere. In Eqn [2], q is the scattered wave vector:

$$q = \frac{4\pi n}{\lambda}\sin(\frac{\theta}{2}), \quad (4)$$

where n is the refractive index of the liquid, θ is the scattering angle and λ is the wavelength of the laser light (Sorensen 2001).

Results and discussion

We determined by mass balance based on the known amino acid sequence of CALB and the elemental analysis of CALBsol for nitrogen (Maxima Laboratories, Inc.) that there were approximately 119 Span 60 molecules per CALB molecule in the $CALB_{sol}$ preparation. Using an approach suggested elsewhere (Paradkar & Dordick 1994) we determined that approximately 251 Span 60 molecules were needed to cover one enzyme molecule. The surface area of the enzyme molecule was calculated using a geometry of $30 \times 40 \times 50$ Å (Uppenberg et al. 1994). Assuming an average area of the hydrophilic head groups of the surfactant molecules of 37.5 $Å^2$ (based on Peltonen et al. 2001) with the surfactant molecule oriented perpendicularly towards the enzyme, the estimated number of 251 Span 60 molecules per enzyme molecule results by dividing the head group area by the enzyme surface area. The considerations above confirmed that our $CALB_{sol}$ preparation was in the surfactant-starved regime relative to complete coverage of all individual enzyme molecules with surfactant molecules.

The maximum solubility for $CALB_{sol}$ in hexane was observed to be approximately 5 mg/mL in water-saturated hexane at 23°C after 2 weeks. A concentration of 0.5 mg/mL was visually observed to dissolve within 10 min above 40°C.

Figure 2 shows a representative example for the intensity autocorrelation function of $CALB_{sol}$/hexane at room temperature. The correlation time was 13 ms corresponding to an average particle radius of 75 nm [eqns 2–4]. The average particle sizes of $CALB_{sol}$ in hexane were found to be about 69 ± 15 nm (room temperature), decreasing with rising temperature to 37 ± 5 nm at 42°C (Figure 3). The fact that larger $CALB_{sol}$ particles were present at the lower temperature might be attributed to agglomeration. We also observed that $CALB_{sol}$ dissolved within minutes at temperatures above 40°C compared with hours to a day at room temperature. This could be interpreted as supporting evidence for the larger particle size detected by DLS at room temperature which would hamper dissolution via mass transfer limitations. It must, however, also be recognized that elevated temperature could be, at least in part, responsible for the more rapid dissolution at higher temperature in addition to differences in particle size.

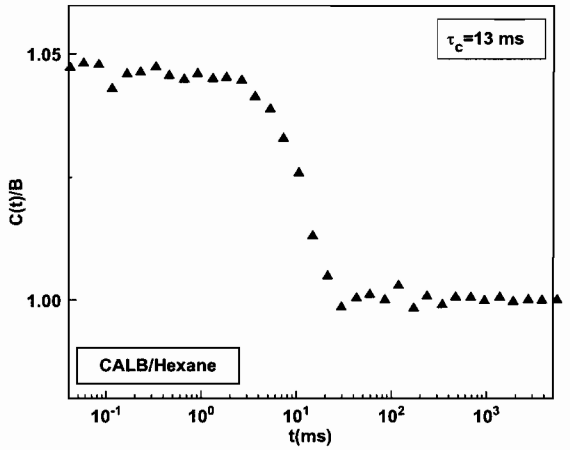

Figure 2. Intensity autocorrelation function of the solubilized CALB/hexane at room temperature.

Some approximate calculations are given here to show that the enzyme molecules are likely solubilized in the form of clusters, rather than as individual molecules. The assumption that the surfactant molecules in $CALB_{sol}$ were associated with CALB appeared reasonable because the surfactant molecules that did not interact with CALB during the solubilization treatment in the aqueous phase would

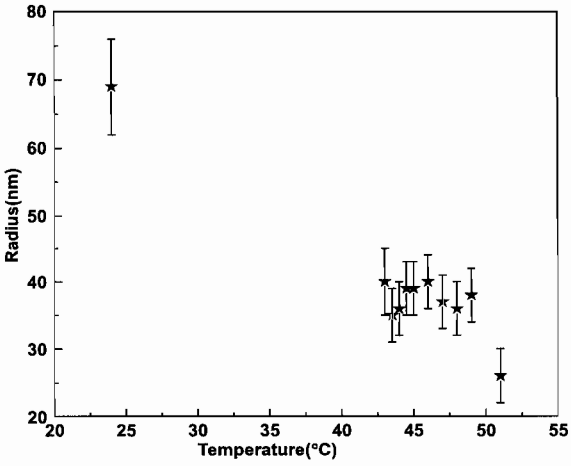

Figure 3. Particle radius for $CALB_{sol}$ as a function of temperature.

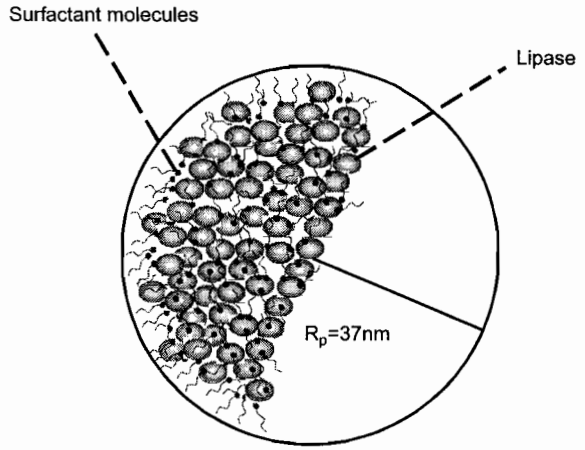

Figure 4. Schematic illustration of a $CALB_{sol}$ cluster (particle radius R_p ~37 nm). The cluster is estimated to contain 1200 CALB molecules with 120 Span 60 molecules per each CALB molecule.

have been discarded with the supernatant after centrifugation.

A suggestion towards the physical presentation of the CALB molecules solubilized by surfactant in hexane under surfactant starved conditions will now be shown. It is assumed here that no solvent is present in the solubilized $CALB_{sol}$ preparation entities which may be reasonable considering that enzymes are not soluble in non-polar solvents such as hexane. The estimated volume for a CALB molecule was $c.6 \times 10^{-26}$ m^3 (see 'Materials and reagents'). Assuming a roughly cylindrical shape for Span 60 molecules, the estimated volume of one molecule is $c.\ 1 \times 10^{-27}$ m^3 as estimated by others (Maruyama et al. 2001). Therefore, the combined volume of 119 Span 60 molecules and one CALB molecule which form a $CALB_{sol}$ particle was estimated to be $c.1.8 \times 10^{-25}$ m^3. A particle with a radius of 37 nm as measured by DLS (see above, for $CALB_{sol}$/hexane above 42°C) would be estimated to accommodate approximately 1200 CALB molecules, each accompanied by 119 surfactant molecules perhaps arranged as illustrated schematically in Figure 4. Combining the volume estimations corresponding with the particle sizes measured via DLS indicates that the enzymes were solubilized in clusters rather than as individual molecules.

Catalytic activity

Unmodified enzymes do not dissolve in hexane and have low catalytic activity (Persson et al. 2002). We were interested not only in investigating the physical result of the solubilization procedure, but also in comparing the catalytic competency of our $CALB_{sol}$ in hexane with a commercial preparation (Novozym 435, CALB immobilized on a solid support).

In our experiments, the $CALB_{sol}$ was used to catalyse a model esterification of geraniol with acetic acid to geranyl acetate, where 1.3 mg of CALB was solubilized in 20 g of hexane at 40°C with initially equimolar substrate concentrations of 0.1 M (Bartling et al. 2001; Kang et al. 2005). Figure 5 shows how the reaction initially progresses by following both one of the reactants (geraniol) and the product (geranyl acetate) by gas chromatography.

We calculated the turnover number k_2 for the first 2 h of reaction (Berg et al. 2001):

$$k_2 = \frac{v_{max}}{[E_T]}, \tag{5}$$

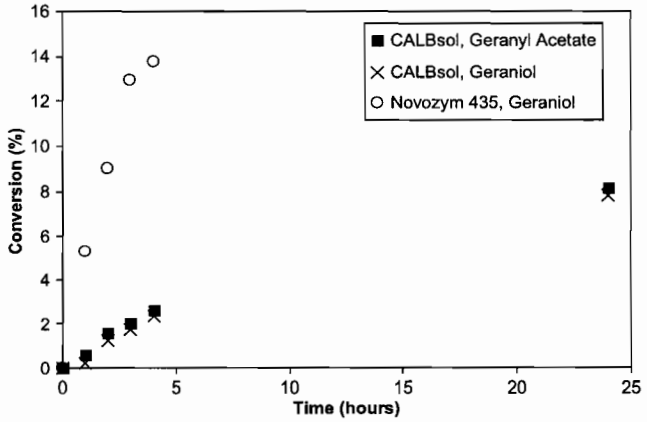

Figure 5. The esterification of geraniol with acidic acid in 19.4 g of hexane catalysed by 1.3 mg solubilized CALB (as $CALB_{sol}$, 40°C, tracked by disappearance of geraniol and appearance of geranyl acetate, initial concentration of reactants 0.1 M). Esterification by Novozym 435 (same conditions, 4.83 mg CALB as Novozym 435) is also shown. The catalytic activity of $CALB_{sol}$ compared with Novozym 435 is lower, but clearly apparent.

where v_{max} is the maximum reaction rate in mol/s and E_T is the total enzyme content in mol. We assume here that the substrate concentration [*S*] is sufficiently large to substitute the measured initial reaction rate (derived from the initial slope of Figure 5) for v_{max}. This yields a conservative estimate of the turnover number, since v_{max} may indeed be higher if [*S*] was perhaps not sufficiently large. It is also assumed that all CALB in either preparation is active. In 2 h, $CALB_{sol}$ produced 4.74×10^{-5} mol geraniol for an average v_{max} of 6.58×10^{-9} L/s. With $[E_T]$ of about 1.58×10^{-8} mol of enzyme contained in the $CALB_{sol}$ used for this reaction the turnover number k_2 for CALB is about 0.42 s^{-1} [eqn 5]. The commercial Novozym 435 reached a k_2 of 1.5 s^{-1} at the same conditions as above assuming that Novozym 435 contains 12.5 wt% lipase. The CALB in $CALB_{sol}$ shows a decreased but significant catalytic activity compared with CALB in the immobilizate Novozym 435.

Conclusions

The solubilization of *Candida antarctica* lipase B (CALB) in hexane with the non-ionic surfactant Span 60 was investigated. The enzyme content for our solubilized CALB (referred to as $CALB_{sol}$) was 40.1 wt%. The particle radius for $CALB_{sol}$ in hexane was found by dynamic light scattering (DLS) to be 37 ± 5 nm between 42° and 50°C. Molecular size calculations and elemental analysis indicated that this corresponded to agglomerates with *c.* 1200 CALB molecules along with *c.* 119 surfactant molecules per CALB molecule. Our results indicated that the surface of the enzyme agglomerates was most likely coated with surfactants, assuming that all surfactant molecules resided at the particle/hexane interface.

$CALB_{sol}$ solubilized in hexane showed catalytic activity in a model esterification reaction with a turnover number of 0.42 s^{-1} at 40°C. DLS proved to be a useful tool to obtain first hand information on the result of solubilization of enzymes in solvents. The catalytic competency of the solubilized CALB in hexane shows another avenue for application of this versatile enzyme.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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