High hydrostatic pressure increased stability and activity of immobilized lipase in hexane

Michael J. Eisenmenger, José I. Reyes-De-Corcuera *

Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 700 Experiment Station Road, Lake Alfred, FL, USA

ARTICLE INFO

Article history:
Received 18 December 2008
Received in revised form 3 March 2009
Accepted 4 March 2009

Keywords:
Immobilized lipase
High hydrostatic pressure
Isoamyl acetate
Enzyme enhancement
Enzyme stabilization
Hexane

ABSTRACT

Lipases are important to high value product synthesis, modification, and enhancement. However, they are often unstable above 40 °C. While most current applications of high hydrostatic pressure (HHP) are for inactivating deleterious enzymes, there is evidence that HHP can stabilize and increase activity of some enzymes. This study examines the apparent kinetics of immobilized lipase-catalyzed synthesis of isoamyl acetate at HHP in hexane. HHP reduced thermal inactivation of lipase by up to 152% after 4 h at 80 °C and 400 MPa when compared to incubations at low pressure. No significant differences were found in activation energy (Ea) at different pressures, irrespectively of the pressurization and heating sequence, and were between 35.7 ± 3.5 and 47.8 ± 8.2 kJ mol⁻¹, depending on the method. In all methods utilized, activity at 63.5 and 80 °C at 400 MPa was greater (from about 20 to 96% increase) than at low pressure. Activity increased by 110% at low pressure versus a 239% increase at 350 MPa when the temperature was increased from 40 to 80 °C. Increasing pressure up to 350 MPa increased lipase activity while pressures greater than 350 MPa maintained or decreased lipase activity. Activation volume (ΔVv) appeared negative between ambient pressure and 200 MPa in contrast to a positive ΔVv between 300 and 600 MPa. Apparent ΔVv was 14.3 ± 1.7 or 15.2 ± 2.2 cm³ mol⁻¹ at 40 or 80 °C, respectively, between 300 and 500 MPa.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Enzymes important to high value product synthesis are often expensive and unstable above 40 °C. Various strategies have been employed to enhance enzyme stability including genetic engineering, immobilization, and operating in non-aqueous media. Isoamyl acetate has been widely used (74,000 kg/year) as a flavor or aroma ingredient in food, cosmetic, and pharmaceutical industries because of its characteristic banana flavor [1] and ability to be considered “natural” [2]. Because conventional flavor generation from plant materials or production via microbial fermentation requires several costly steps an alternative enzymatic method using lipases has become the focus of many studies [3–10]. Lipases are widely used in industrial applications because of their high enantioselectivity, wide range of substrates, thermal stability, and stability in organic solvents [11]. Isoamyl acetate esterification catalyzed by lipases from various sources has been studied in a wide variety of solvents [5,6,10], kinetic studies have proposed a Ping-Pong Bi–Bi mechanism [3], and operating conditions such as acyl donor, type of lipase, and temperature have been optimized [5]. However, the application of high hydrostatic pressure (HHP) as a reaction parameter has not been explored in hexane.

While HHP is effective at inactivating various deleterious enzymes [12–18], its effects on enzyme stabilization and activation have been documented relatively little [19–23]. HHP has been shown to stabilize or activate chymotrypsin [24], naringinase [25,26], polyphenol oxidase [17], pectin methylesterase [17,27,28], and more extensively lipases in dense gases and solvent free systems [6,29–34] among many others. No work to date has focused on investigating HHP enhanced stability or increased activity of lipase-catalyzed synthesis of isoamyl acetate in an organic solvent.

Pressure and heat are generally thought to be antagonistic factors in molecular terms (from the principle of microscopic ordering, an increase in pressure at constant temperature leads to an ordering of molecules or a decrease in the entropy of the system) and with regards to enzyme conformation [21–23,35]. This antagonistic relationship results in an elliptical pressure versus temperature diagram with native and denatured protein regions inside and outside of the ellipse, respectively [21]. During the pressure and temperature come-up time the enzyme may undergo irreversible denaturation that results in a decrease in activity. While these antagonistic effects have been previously explored and discussed [21–23,35] no work to date has compared the different effects of applying HHP first then heating, heating first then applying HHP, or attempted to apply HHP and heat simultaneously and assessed the effects on rate constant and activation energy, in particular for lipase-catalyzed synthesis of isoamyl acetate in organic solvent.
Operating at HHP offers other less complex advantages over conventional synthesis at ambient pressures. For example, Candida antarctica lipase B (CALB) is a relatively heat-tolerant enzyme that is active up to 100 °C [5]. Furthermore, several other extremely heat-tolerant (stable up to 150 °C) enzymes have been discovered and may have a potential application in biocatalysis in organic media [36–38]. However, the boiling point of most organic solvents is well below 100 °C. For example hexane boils at 68.7 °C (at 0.1 MPa) which restricts studies in hexane to be explored below that temperature. As pressure is raised the boiling point of organic solvents also increases allowing studies to examine activity above conventional temperatures at low pressure [39]. The ability to operate with organic or volatile solvents at temperatures above their conventional boiling point is a significant yet often overlooked advantage of HHP.

The cost of high pressure processing (HPP) has decreased over the last decade and become more widely implemented particularly in the food industry. In 2007 there were about 120 HPP industrial installations operating worldwide [40] with 80% of their equipment installed since 2000. HPP food has become a two billion dollar global market and is expected to comprise 450 million pounds/year in 2009 [41]. As demand for HPP equipment grows, innovation is expected to continue to reduce capital and operating costs [40]. Although HPP of bulk foods is currently more widespread, the much higher profit-margin sector of enzyme catalyzed flavor synthesis may have greater potential for adopting HPP.

The objective of this research was to characterize the effects of HHP and temperature on lipase activity and stability during the synthesis of isoamyl acetate in hexane.

2. Experimental

2.1. Materials

Lipase (Novozyme 435® E.C. 3.1.1.3) from C. antarctica lipase B (CALB) expressed in Aspergillus oryze immobilized on a macroporous acrylic resin (13,100 PLU/g) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Isoamyl alcohol, glacial acetic acid, and HPLC grade hexane were obtained from Fisher Scientific (Pittsburg, PA, USA). All solvents and substrates were held at −10 °C or on ice while preparing for assay. Reaction vials were made using 3-mL syringes with Luer-Lock® tips (BD Franklin Lakes, NJ, USA) which allowed substrate insertion with an opposing plunger while preventing solvent, substrate, or enzyme leaching during pressurization.

The HHP system consisted of a high pressure reactor (model U111), a high pressure micropump (model MPS), and a pump controller (MPS micropump control unit) all from Unipress Equipment (Warsaw, Poland). The reactor was temperature-controlled with a water jacket alternatively fed by two water baths (Isotemp 3016D; unit) all from Unipress Equipment (Warsaw, Poland). Associated with HHP systems upon pressurization and depressurization were not significant factors as the temperature controlled jacket limited temperature fluctuations to less than 2 °C. The high pressure reactor was closed pressure increased simultaneously with temperature. When temperature reached 68.7 °C (hexane boiling point at ambient pressure) pressure has already reached 5 MPa. In other words, during the heating of the reaction cell, because of the simultaneous increase in pressure, hexane never reached the boiling point. Within the context of this study, pressures below 10 MPa, reached under these conditions, are referred as “low pressures”. Pressure and temperature effects on the reaction, in the absence of enzyme, were ruled out when no significant reaction progress was observed at pressures up to 700 MPa and 80 °C for 4 h (data not shown) which was similar to previous studies [42] where isoamyl acetate production was negligible in absence of a catalyst. Native lipase activity was determined by assaying lipase that has not been exposed to temperatures above recommended storage conditions (5 °C). Percent relative activity was determined by comparing the initial rate of the observed reaction to that of the native enzyme (control). Samples were treated and assayed in a randomized block design, blocked by temperature and incubation time while pressure varied. Significant differences in treatments were determined using analysis of variance (ANOVA) and Tukey’s pair-wise comparison at α = 0.05 for enzyme treatments and controls. Statistical analysis was done using SAS software (Cary, NC, USA).

2.2. Methods

2.2.1. Effect of HHP on lipase stability

Enzyme was weighed (10 mg or ~128 Propyl Laureate Units) into the reaction vial. One milliliter of hexane and a miniature stir bar were added to the enzyme. The reaction vial plunger was moved into position to eliminate air bubbles then sealed with a Luer-lock® plug. The reaction vial was then placed in the high pressure reaction chamber being held at 10 °C. Polydimethylsiloxane silicone liquid (Accumetric Inc., Elizabethtown, KY, USA) was added as hydraulic fluid to fill the reactor. The reactor was sealed and pressurized. After pressure reached the set point, temperature was adjusted to 80 °C. Incubations were held at pressures from 10 to 700 MPa for 4 h. Upon completion of incubation, the temperature was returned to 10 °C, and the reactor was depressurized and opened. The reaction vial was withdrawn. Lipase activity was determined by monitoring the reaction progress of the esterification of isoamyl alcohol and acetic acid to form isoamyl acetate as previously described [3,5] and shown as:

\[
\text{Isoamyl Alcohol} + \text{Acetic Acid} \rightarrow \text{Isoamyl Acetate}
\]

The esterification reaction was initiated by addition of 1 mL 0.12 M isoamyl alcohol in hexane and 1 mL of 0.12 M acetic acid in hexane combined to make 0.06 M substrate solution into the reaction vial through the Luer-lock®. The reaction mixture was incubated at 10 °C and stirred continuously. A 1-μL aliquot was drawn for GC-FID analysis every 10 min. The apparent initial rate was determined by linear regression of the progress curve in the linear range (time ≤ 30 min). Peak identification was determined using pure standards. Reaction stoichiometry has been recently reviewed and confirmed [3,5,7], which allowed kinetic analysis to be conducted by following product formation. Adiabatic heating or cooling effects commonly associated with HHP systems upon pressurization and depressurization were not significant factors as the temperature controlled jacket limited temperature fluctuations to less than 2 °C. Because the high pressure reactor was closed pressure increased simultaneously with temperature. When temperature reached 68.7 °C (hexane boiling point at ambient pressure) pressure had already reached 5 MPa.

Fig. 1. Schematic representation of the high hydrostatic pressure system.
and activation energies. Application sequences were pressurization first, simultaneous heating and pressurization, and heating first as shown in Fig. 2. In all cases, incubation time began when the desired pressure was achieved (±10 MPa) and at least 90% of the difference between cold temperature and incubation temperature were reached. Controls (100% activity) correspond to the activity after reaching set point temperature and pressure then depressurization and cooling with no incubation period. Apparent initial rate was determined by mixing 2.5 ± 0.1 mg immobilized lipase and 2 mL of isoamyl alcohol and acetic acid 0.06 M in hexane in the reaction vial and immediately inserted into the reactor. Pressure (0.1–400 MPa) and temperature (36, 42, 48.9, 56.2, 63.5, 71.8, 80 °C) were adjusted according to method of application as previously discussed and shown in Fig. 2 and compared to assays at low pressure. The reaction vial was held at 5 ± 0.1 °C prior to and after incubation conditions. During incubation the reaction mixture was stirred with the magnetic stir bar. Upon completion of incubation cycle the reaction vial was removed and a 1-μL aliquot was taken for GC-FID analysis at time intervals of 0, 2.5, 5, 7.5, and 10 min. Rate was determined from the slope of the linear regression (R² > 0.95) of the time course production of isoamyl acetate by fitting product concentration versus time. Reaction rate was expressed as concentration (mml/L. °C) isoamyl acetate generated per second. Arrhenius plots were used to determine the initial reaction rate dependence on temperature between 36 and 80 °C and calculate Ea. Statistical comparisons of treatment methods were conducted using SAS statistical software (Cary, NC, USA) utilizing GLM regression analysis.

### 2.2.3. Activation volume

Pressure effects on initial rate were examined at 0.1–600 MPa at 40 or 80 °C. Initial rate was determined by mixing 2.5 ± 0.1 mg immobilized lipase and 2 mL of isoamyl alcohol and acetic acid 0.06 M in hexane in the reaction vial and immediately inserted into the reactor. Rate was determined from the slope of the linear regression (R² > 0.95) of the time course production of isoamyl acetate by fitting product concentration versus time. Reaction rate was expressed as concentration (mml/L. °C) isoamyl acetate generated per second. Pressure and temperature treatments were applied using the "pressure-first" method described in Section 2.2.2. The effects of pressure on enzyme activity are described by an overall activation volume (ΔV⁺) determined from Arrhenius's equation:

\[
\frac{\beta \ln k}{\beta p} = \frac{-\Delta V^+}{R T}
\]

where k is the equilibrium constant, p the pressure, T the absolute temperature, R the gas constant and ΔV⁺ is the activation volume [43].

### 2.2.4. Electron micrographs of immobilization beads

SEM was utilized to examine the solvent, temperature, and pressure effects on the immobilization support (macroporous acrylic resin) shape and conformation as well as the surface texture. Samples were dried at 40 °C for 30 min before a 90-s application of gold palladium coating. Incubation treatments included a control (no treatment), exposure to hexane for 4 h, exposure to hexane and 80 °C for 4 h, and exposure to hexane at 80 °C and 400 MPa for 4 h, and after assaying with and without hexane, heat, and pressure treatment.

### 3. Results and discussion

#### 3.1. Effect of HHP on lipase stability

Immobilized lipase was incubated at 80 °C and from 10 to 700 MPa for 4 h. Results are shown in Fig. 3. Residual activity at ambient pressure after the 4-h treatment was 40%. Relative residual activity increased with pressure until reaching a maximum at around 400 MPa where residual activity was around 99%. Pair-wise comparison of enzyme treatment and controls using Tukey’s test (α = 0.05) allowed determination of significant differences between pressure treatments. Residual activity was significantly higher above 300 MPa compared to at 10 MPa. Residual activity at 400, 600, and 700 MPa was significantly higher than at 100 MPa. Compared to incubations at ambient pressure at 80 °C for 4 h, at 400 MPa thermal inactivation of lipase was reduced by up to 152%. The confidence intervals are larger than desired due to experimental error associated with difficulties in measuring small quantities of immobilized enzyme (10 mg) beads with different surface area on which lipase is bound [44]. The largest surface area of the immobilization bead was 4-fold that of the smallest. Therefore, changes in surface area greatly affect the total number of enzyme units per weight of beads. Residual activity near or slightly above 100% at 400 MPa may be due to pressure- and heat-induced conformational changes in the lipase allowing greater activity post-incubation but most probably are simply the result of experimental error described above. In order to clarify this issue in situ conformational studies are needed to observe structural changes which may be occurring during come-up time, incubation time, and come-down time. Similar studies have been conducted at elevated pressure using fluorescence spectroscopy [45], derivative spectroscopy [46], X-ray scattering [47], and quantitative Raman spectroscopy [48]. To conduct these studies with CALB, the enzyme must be solubilized in hexane using a synthetic detergent according to Tsuzuki and Suzuki [49], and require different specialized HHP optical cells depending on the method used.

Pressure-induced stabilization was also shown for a similar from Rhizomucor miehei lipase in soluble form in aqueous solution as well as invertase from Saccharomyces cerevisiae [50]. At denaturing temperatures (50, 55, and 60 °C) application of pressure protected the

---

Fig. 2. Temperature (○) and pressure (▲) profiles for (A) “pressure-first”, (B) “simultaneous”, and (C) “heat-first” method for a 10-min incubation at 80 °C and 400 MPa.

---

Fig. 3. Effect of high pressure on enzyme stability after incubation for 4 h at 80 °C. Assayed with 10 ± 0.5 mg lipase, 0.06 M substrate. Error bars represent a 95% confidence interval using analysis of variance.
lipase at a range of 50–350 MPa by increasing residual activity from about 82 to 95% at 50 °C, from about 32 to 70% at 55 °C, and from 0 to about 35% at 60 °C [50]. The protective effect of pressure increased with pressure up to 150–250 MPa with more pronounced stabilization effects as temperature increased from 40 to 60 °C. In the same study, it was observed that invertase half-life increased until pressure reached 200 MPa, meaning that the enzyme was protected by pressure in the range of 50–200 MPa and beyond 200 MPa residual activity decreased [50]. This phenomenon is also similar to that of pectin methyl esterase (PME) which showed an increase in optimal temperature from 45 to 55 °C as the pressure increased from 0.1 MPa to 300–500 MPa [28].

These stability studies indicate that pressure significantly stabilizes the immobilized lipase up to 400 MPa. Recently, the activity of lipases from porcine pancreas, C. antarctica, Candida cylindracea (immobilized), Penicillium roqueforti, Aspergillus niger, Rhizopus arhizus, Mucor miehei, and Pseudomonas cepacia was studied after hydrolysis under supercritical carbon dioxide (SC-CO₂) at 40 °C and 15 MPa [51]. All lipases tested had increased relative activity with the greatest increase from Rhizopus arhizus lipase which was increased by more than 50 times while C. antarctica lipase activity increased from 0.32 to 5.97 U g⁻¹ after treatment in SC-CO₂ and up to 21.00 U g⁻¹ during exposure to SC-CO₂. However, these results contrast to previous studies of lipase in SC-CO₂ and compressed n-butane which showed increased activity as pressure increased up to 35 MPa [30,31,33] with a precipitous drop beyond that.

Although the mechanism of HHP-induced stabilization has yet to be fully elucidated, it appears that pressure effects on intramolecular interactions, hydration of charged groups, disruption of bound water, and stabilization of hydrogen bonds may all play some role. According to Mozhave et al. [24], the antagonistic effects of pressure and temperature rests upon counteracting effects on the ability of functional groups to interact with water. Pressure enhances the hydration of charged groups, whereas the hydration of charged groups is loosened at high temperature [52]. Furthermore, opposing effects of pressure and temperature with respect to hydrophobic interactions and hydrogen bonds offer a possible explanation for pressure stabilization of enzymes against thermal inactivation [53]. Endothermic hydrophobic interactions are known to be enhanced at elevated temperatures, being maximal at about 60–70 °C and thereafter decreasing because of a gradual breakdown of the water structure [54]. Hydrogen bridges are generally known to be stabilized by pressure and destabilized by heat [54,55]. These cumulative antagonistic effects of pressure and heat may offer explanation of pressure-induced resistance to thermal inactivation.

Pressure-induced stabilization is contrary to pressure effects of a similar lipase from R. miehei in aqueous solution. According to Noel and Combes [45] pressure treatment under 450–500 MPa at 25 °C had equivalent inactivation rates as thermal treatment at 50–55 °C. These apparent conflicting results may be contributed to various different incubation conditions, mainly enzyme immobilization and solvent polarity as well as inherent differences in the lipases tertiary and secondary structures. However, in the same study Noel and Combes [45] concluded that lipase has a high stability under pressure as represented by a slight change in enzyme volume (0.2%) in comparison to initial volume of the native protein. Furthermore, it was concluded that the application of high pressure combined or as an alternative to high temperature is of immediate relevance to modulate enzymatic activity [45].

3.2 Effect of HHP on lipase activity

3.2.1 Comparison of pressurization and heating methods on activation energy

Initial rate of isoamyl acetate production using each method at 400 MPa and 36–80 °C was compared to assays at low pressure. Apparent initial rate results are shown in Table 1 and summarized with percent change as compared to rates at low pressure. In all sequences activity at 71.8 or 80 °C and 400 MPa was greater than at low pressure by 19.8–96.1% depending on the method and temperature. Activation energy (E_a) was determined for each of three methods. At temperatures above 63.5 °C Arrhenius plots deviated from linearity, indicating that unlike in the stability study in Section 3.1, in the presence of substrate, lipase inactivation may be occurring. In the time frame of these experiments the rates of reaction still increased with temperature. In contrast, at ambient pressure, activity decreased with increasing temperature above 63.5 °C. Therefore, E_a was determined in the range of 36–63.5 °C. E_a at low pressure and was 42.7 ± 3.80 kJ mol⁻¹. The pressure-first, simultaneous, and heat-first methods had E_a of 42.36 ± 6.9, 47.8 ± 8.16, and 35.7 ± 3.5 kJ mol⁻¹ respectively. No significant differences were found in E_a at any pressure tested irrespectively of the pressurization and heating sequence. This contrasts with the findings of Weemaes et al. [53] who found that E_a varied with hydrostatic pressure for polyphenoloxidase, whereby it was found that activation energy decreased with increasing pressure. The heat-first method consistently resulted in slightly higher rates than all other methods tested at all temperatures as shown in Table 1. This is most likely because temperature was consistently higher at the beginning of the incubation time using the heat-first method, while incubation time started when temperature reached 90% of the set point for the other two methods while for the heat-first method, pressurization time allowed heating to about 95% of the set point. CALB in n-hexane using acetax anhydride (a more effective acyl donor) resulted in lower E_a (11.3 kJ mol⁻¹) [5] for synthesis of isoamyl acetate. E_a was slightly higher to that reported in a solvent free system (28.7 kJ mol⁻¹) [56] and in biphasic-ionic liquid (27.3 kJ mol⁻¹) [10]. These results are similar to other ester synthesis reactions catalyzed by CALB; including butyl butyrate (32 kJ mol⁻¹) in SC-CO₂ [57] and tetrahydrofurufuryl butyrate (47 kJ mol⁻¹) [58], oleyl oleate (37 kJ mol⁻¹) [59], butyl laurate (45 kJ mol⁻¹) [59], butyl oleate (37 kJ mol⁻¹) [59], and oleyl butanoate in hexane (39 kJ mol⁻¹) [59].

Several enzymes have shown enhanced activity while or after exposure to HHP; including polyphenoloxidase [60,61], pectin methylesterase [27,28,α-chymotrypsin [24], dehydrogenase [62,63], protease [64], α-amylase [65], peroxidase [61], thermolysin [66], and pepsin [66] among others. Several reasons for pressure-induced changes in the rate of enzyme catalyzed reactions have been offered and are classified into three groups: (1) changes in the structure of an enzyme; (2) changes in the reaction mechanisms; for example, a change in the rate-limiting step; and (3) changes in the substrate or solvent thus affecting enzyme structure or the rate limiting step.

Enzymatic conformation is affected by both pressure and temperature, thus affecting activity. CALB is a monomeric enzyme and there is evidence that activities of monomeric enzymes are stimulated while the activities of multimeric enzymes are inhibited by application of high hydrostatic pressure [67]. However, at least 15 dimeric and tetrameric enzymes that are activated by pressure have been reported [68]. Noel and Combes [45] compared conformational changes at elevated pressure (300–500 MPa) to those at elevated temperatures (40–60 °C) by measuring emission spectrum at 290 and 350 nm for R. miehei lipase. Conformational changes induced by pressure were attributed to aggregation (increased fluorescence at 290) and were different from those induced by temperature, attributed to unfolding (increased fluorescence at 350). In both cases decreased activity was reported.
Table 1

Initial rate and percent change using various "pressure first", "heat first", and "simultaneous" methods of applying heat and pressure as compared to assays at low pressure.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Low pressure</th>
<th>Heat first (400 MPa)</th>
<th>Simultaneous (400 MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate (mmol L⁻¹ s⁻¹)</td>
<td>Pressure* (MPa)</td>
<td>Initial rate (mmol L⁻¹ s⁻¹)</td>
</tr>
<tr>
<td>36.0</td>
<td>5.31 × 10⁻³</td>
<td>0.1</td>
<td>4.49 × 10⁻³</td>
</tr>
<tr>
<td>42.0</td>
<td>7.45 × 10⁻³</td>
<td>1</td>
<td>4.50 × 10⁻³</td>
</tr>
<tr>
<td>48.9</td>
<td>1.00 × 10⁻²</td>
<td>2</td>
<td>9.32 × 10⁻³</td>
</tr>
<tr>
<td>56.2</td>
<td>1.69 × 10⁻²</td>
<td>6</td>
<td>1.02 × 10⁻²</td>
</tr>
<tr>
<td>63.5</td>
<td>1.94 × 10⁻²</td>
<td>7</td>
<td>1.62 × 10⁻²</td>
</tr>
<tr>
<td>71.8</td>
<td>1.60 × 10⁻²</td>
<td>8</td>
<td>1.91 × 10⁻²</td>
</tr>
<tr>
<td>80.0</td>
<td>1.57 × 10⁻²</td>
<td>10</td>
<td>2.49 × 10⁻²</td>
</tr>
</tbody>
</table>

Bold font indicates a pressure-induced positive increase in activity.

* Increasing pressure for “low pressure” treatment is due to heating of the sealed HHP system with the pump off.

was higher at 80 °C than at 40 °C at all pressures. At 80 °C V_max with pressure in the range of 0.1–300 MPa followed by a decrease in the range of 350–600 MPa. At 40 °C V_max increased with pressure in the range of 0.1–100 MPa followed by a decrease in the range of 200–600 MPa. Activities at 100–400 MPa at 40 °C and at 100–450 MPa were higher than at low pressure. Maximum rate of reaction was 2.09 × 10⁻³ at 300 MPa and 80 °C. This pressure was below that of maximum stability (400 MPa) discussed in Section 3.1. Reversible enzyme denaturation may account for this decrease because, unlike in the stability studies where pressurization was done in the absence of substrate and activity assays were carried out ex-situ, the progress of this reaction occurred under pressure in the presence of substrate. The relationship between pressure and the logarithm of V_max was linear between 300 and 500 MPa at 40 and 80 °C. Therefore, the effect of pressure in reaction rate was described by Eyring equation as shown in Fig 5 and ΔV⁰ was determined. ΔV⁰ slightly increased with temperature from 14.3 ± 1.7 to 15.2 ± 2.2 cm³ mol⁻¹ at 40 or 80 °C, between 300 and 500 MPa. These results were not significantly different. Interestingly, apparent ΔV⁰ was negative from 0.1 to 200 MPa at 80 °C and from 0.1 to 100 MPa at 40 °C. At pressure below 400 MPa inactivation occurred at 80 °C as previously shown in Fig. 3. Therefore, at 80 °C below 400 MPa the apparent negative ΔV⁰ may be attributed to partial enzyme inactivation. Pressure-induced stabilization offers a possible explanation for the increased activity at 80 °C, but not at 40 °C where the lipase is considered stable [5]. Therefore, both enzyme stabilization and pressure activation might be occurring simultaneously. Recently carrot PME showed very similar effects while at range of temperature (30–60 °C) and pressures (0.1–600 MPa) [69]. The PME showed a negative ΔV⁰ below 400 MPa at temperature greater than 60 °C and a positive ΔV⁰ above 400 MPa at all temperatures tested [69].

To the best of our knowledge there are no other reports on the effects of HHP on CALB in organic media. However, the effect of high pressure in SC-CO₂ on the isoamyl acetate synthesis from isoamyl alcohol using CALB was reported by Romero et al. [6]. Pressure had no significant influence on esterification of isoamyl acetate between 8 and 30 MPa at 40 °C. This may be attributed to the relatively low and narrow pressure range used by that group. In another study, P. cepacia lipase (PCL)-catalyzed transesterification of 1-phenylethanol with vinyl acetate in SC-CO₂ showed an increase in catalytic efficiency up to 15 MPa. However, ΔV⁰ did vary with pressure range used, reaching a maximum negative value of −1340 cm³ mol⁻¹ at 7.4 MPa [70]. High negative ΔV⁰ would be expected in SC-CO₂ at this narrow pressure range (0.1–15 MPa) due to changes in solvent phase and density as well as solubility and partitioning of substrates but not as direct effect on enzyme activity.

Lipase activity may be higher between 100 and about 350 MPa compared to at low pressure due to a shift in the tertiary structure surrounding the active site allowing greater substrate interaction. Crystallographic and molecular-modeling studies of CALB determined that lipase belongs to the α/β hydrolase fold family with a catalytic triad consisting of Ser, His, and Asp/Glu [71]. Early X-ray diffraction data suggests that a lipase’s helical part of the α-helix near the active site forms a “lid” that rotates almost 90°, from lying flat on the surface to extending nearly perpendicular to it on rearrangement to the open conformation [72]. However, recent studies conducted in aqueous media have concluded that in contrast to most lipases, CALB may not have a “lid” covering the entrance to the active site and shows no interfacial activation [73] as compared to C. antarctica lipase A (CALA) which does possess the characteristic “lid” structure and shows interfacial activation [74]. Furthermore, flexibility of CALB is reduced in organic solvents, and is limited to: (1) a short α-helix which form the entrance to the active site, (2) three surface loops, (3) the medium binding pocket for secondary alcohols, (4) the acyl binding pocket, and (5) the large binding pocket for secondary alcohols [73]. Flexibility limited to these five elements vital to catalysis may offer possible explanation for relatively high pressure stability, while still allowing modification of activity. Although flexibility is limited in organic solvents, to the
best of our knowledge the effects of pressure on the flexibility of these five elements or other modifications of the active site have not been explored in organic solvents at HHP.

Pressure effects on proteins and enzymatic reactions have been discussed and reviewed in depth over the past 20 years [20–23,35,52,75,76]. It is understood that pressure effects on catalysis reactions are related to Le Chatelier’s principle: elevated pressures favor changes that reduce a system’s overall volume. Thus, by comparing the total volumes of the reactants versus products, of the ground state versus activated state, or of the dissociated versus bound complex, the effect of pressure on reaction equilibria or rates can be estimated [35]. HHP increases substrate concentration as a result of a decrease in volume thus increasing reaction rate. Concurrently HHP increases solvent viscosity hindering reaction rate. In this study viscosity effects may be negligible due to constant vigorous agitation during incubation and activity assays. In contrast to simple chemical reactions, it is difficult to give a precise interpretation of calculated $\Delta V^\neq$ for enzymatic synthesis reactions because pressure affects several factors including: enzyme conformation, enzyme solvation (interaction with surrounding medium, other proteins, water, ions, etc.), immobilization matrix, etc. Furthermore, neither positive nor negative activation volumes seem to be predominant in enzyme reactions. Typically, however, $\Delta V^\neq$ ranges from −70 to 60 cm$^3$ mol$^{-1}$ with most being less than 30 cm$^3$ mol$^{-1}$ [35] which is in agreement with the findings of this study.

The pressure dependence of the solubility parameter and molar volume (among others) of hexane has been recently described at pressures up to 300 MPa at 30°C. Pressure monotonically increased the solubility parameter (∼20%) and decreased the molar volume (16%) [77]. It was determined that use of more non-polar solvents on the synthesis of isoamyl acetate results in better esterification as they preserve the catalytic activity without disturbing the microaqueous layer of the enzyme [8]. Pressure increases hexane’s solubility parameter thus may be antagonistic to the esterification of isoamyl acetate because esterification is favored in less polar solvents. However, the flexibility of CALB is increased with increasing polarity of the organic solvent [73]. Therefore, pressure may increase flexibility of CALB.

Lipase activity in organic solvents was correlated with the solvent polarity [78]. However, it has also been reported that lipase activity might be affected by the solvent without correlation to the polarity [79,80]. Lipases that are active in organic solvents retain their native structure upon transfer from water to organic solvents [70,71]. However, it is essential to have small amounts of water to maintain stability and flexibility of lipases in organic solvent. Thus, enzyme-bound water is essential for catalysis and serves as a lubricant for the enzyme. In contrast, fully dry enzymes are inactive and enzymes in organic solvents with high amounts of water show denaturation [81,82]. Since pressure has been shown to enhance the hydration of charged groups one can hypothesize that pressure helps to maintain bound water, therefore enhancing activity. These pressure dependent changes of the reaction medium and their effects on lipase stability and activity remain unclear and need further exploration.

3.2.3. Electron microscope examination of immobilization beads

In an effort to isolate HHP effects on lipase versus the effects on the immobilization support, SEM images were taken of the immobilized lipase at various stages throughout the treatments. It was hypothesized that increased activity may be generated from deformations, cracking, or splitting of the immobilization support. This was not found to be the case as Fig. 6 shows the SEM of immobilization supports (macroporous acrylic resin beads) before (Fig. 6A) and after (Fig. 6B) exposure to hexane at 80°C and 700 MPa for 4 h magnified to examine shape of resin bead and bead surface. The immobilization supports were also examined after assay without incubation (Fig. 6C) and after assay at 80°C and 700 MPa for 15 min (Fig. 6D). These results indicate that no visible change has

![Fig. 6. Scanning electron micrographs of the surface of Lipozyme® (Novozyme L4777) immobilized on acrylic resin (A) before and (B) after exposure to hexane solvent at 80°C and 700 MPa for 4 h and after assay (C) without incubation and (D) with incubation at 80°C and 700 MPa for 4 h treatment. Inserts illustrate the retention of bead integrity after different treatments.](image-url)
occurred in either the overall shape or in the surface texture of the immobilization bead as compared to the control and to previous studies [44]. Surface differences do appear in Fig. 6A and B due to orientation of the bead during imaging. Also, differences can be seen when comparing Fig. 6A and B, and C and D due to residue on the surface. These visible residues may be attributed to assay effects on the immobilization support. There was a large variation in size of immobilized beads. The largest bead was approximately 500 μm while the smallest was approximately 125 μm. This variation in diameter corresponds to a 4-fold variation in surface area. Lastly, presence of some cracked or broken beads were seen equally in both controls and treatments. The lack of a significant change in bead shape or surface contour adds further evidence confirming that pressure, temperature, and solvent effects are acting directly on the enzyme and not physically distorting the immobilization support which could result in apparent improved activity. However, it remains a possibility that the immobilization support is distorted while under pressure and returns to original conformation upon depressurization. Changes in the structural integrity of immobilization supports may be monitored in situ at elevated pressure by using high pressure optical cells with diamond or sapphire windows [47,48] which allow continuous monitoring.

4. Conclusions

Previous studies [3,5,7] indicate that the optimal conditions for isoamyl acetate formation using lipase in hexane at atmospheric pressure was 43.2 °C. This study indicates the optimal thermal conditions of this reaction may be dramatically increased by the implementation of HHP. The results of this study suggest that the stabilization and catalytic improvement effects of HHP have potential for various applications to lipase-catalyzed production of isoamyl acetate.

While enzymatic enhancement clearly occurs up to 400 MPa, the effects beyond this pressure are not fully understood. Many areas remain to be investigated including the mechanism of HHP-induced stabilization and increased or decreased activity, HHP effect on other immobilized and free lipases, effects of HHP during extended incubation times (>4 h), and a complete optimization of HHP utilization for isoamyl acetate synthesis. While the relationship between elevated pressure and temperature effects on lipase remain to be completely resolved, it is clear that HHP is a useful reaction parameter for improving lipase catalysis.

This study illustrates the complexity of enzyme kinetics at a range of pressures. As opposed to simple chemical catalysis, the apparent activation volume may appear positive or negative depending on the pressure effects on enzyme conformation, temperature—pressure antagonistic effects, solvation effects, as well as pressure effects on the chemical equilibrium.

Acknowledgments

This project was supported by a Research Innovation Grant from the University of Florida Institute of Food and Agricultural Sciences. The authors are grateful for the assistance of Mrs. Diane Achor of the Citrus Research and Education Center, Electron Microscopy Laboratory, and to James Colec of the Institute of Food and Agricultural Sciences statistical group.

References


M. J. Eisenmenger, J. I. Reyes-De-Corcuera / *Enzyme and Microbial Technology* 45 (2009) 118–125


