ENHANCEMENT OF LIPASE CATALYZED ISOAMYL ACETATE SYNTHESIS BY HIGH HYDROSTATIC PRESSURE AND USE OF ALTERNATIVE SOLVENTS

By

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To my lovely wife, mother, father, and all my family and friends who helped, encouraged, and guided me through my pursuit of a higher education. Without whom I could not have achieved my goals.
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<table>
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<th>Abbreviation</th>
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<tr>
<td>HP</td>
<td>High pressure</td>
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<tr>
<td>HHP</td>
<td>High hydrostatic pressure</td>
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<tr>
<td>HPT</td>
<td>High pressure treatment</td>
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<tr>
<td>SC-CO₂</td>
<td>Supercritical carbon dioxide</td>
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<tr>
<td>BPTI</td>
<td>Bovine pancreatic trypsin inhibitor</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>GAPDHs</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GDH</td>
<td>Glutamate dehydrogenase</td>
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<tr>
<td>YADH</td>
<td>Yeast alcohol dehydrogenase</td>
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<tr>
<td>TBADH</td>
<td>Thermoanaerobium brockii alcohol dehydrogenase</td>
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<tr>
<td>POD</td>
<td>Peroxidase</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
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<tr>
<td>PPO</td>
<td>Polyphenoloxidase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PME</td>
<td>Pectin methylesterase</td>
</tr>
<tr>
<td>SCSF5</td>
<td>Sulfur hexafluoride</td>
</tr>
<tr>
<td>CALB</td>
<td>Candida antarctica lipase B</td>
</tr>
<tr>
<td>CALA</td>
<td>Candida antarctica lipase A</td>
</tr>
<tr>
<td>Eₐ</td>
<td>Activation Energy</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>PG</td>
<td>Polygalacturaonase</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
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<tr>
<td>CT</td>
<td>Alpha-chymotrypsin</td>
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BLG  Beta-lactoglobulin
While most current applications of high pressure (HP) are for inactivating deleterious enzymes, there is evidence that high pressure can induce stabilization and activation of some enzymes. Various other strategies have been employed to enhance enzyme stability, including; genetic engineering, immobilization, and operating in non-aqueous media. While each of these strategies has provided varying degrees of stability or activity enhancement, the application of high pressure may be a complementary, synergistic, or an additive enzyme enhancement technique. Over 25 enzymes that have exhibited high pressure stabilization and/or activation were compiled. Each enzyme discussed responds differently to high pressure depending on the pressure range, temperature, source, solvent or media, and substrate. Possible mechanisms for pressure-induced stabilization and activation are discussed and compared with current enzyme enhancement techniques.

Lipases are the most widely used enzyme in high value product synthesis, modification, and enhancement. However, they are often unstable above 40 °C. Isoamyl acetate has a banana flavor and is widely used. When synthesized from a lipase-
catalyzed reaction it can be considered a “natural” flavor ingredient. 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, regardless 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 (ΔV‡) appeared negative between ambient pressure and 200 MPa in contrast to a positive ΔV‡ between 300 and 600 MPa. Apparent ΔV‡ was 14.3 or 15.2 ± 2.2 cm³ mol⁻¹ at 40 or 80 °C, respectively, between 300 and 500 MPa.

This study also examines further the negative and positive activation volumes (ΔV‡) and Michaelis-Menten parameters (K_M and V_max) of immobilized lipase-catalyzed synthesis of isoamyl acetate at HHP in hexane. At 80 °C V_max increased (negative ΔV‡) from 10 to 250 MPa, remained relatively constant between 250 and 350 MPa, then decreased (positive ΔV‡) from 350 to 600 MPa. At 40 °C V_max increased (negative ΔV‡) from 1 to 100 MPa, remained relatively constant between 100 to 200 MPa, and then decreased (positive ΔV‡) from 200 to 600 MPa. Temperature affected ΔV‡ at low pressure but did not at high pressure; indicating that pressure induced activation is.
affected by temperature while inactivation is not. Pressure significantly increased $V_{\text{max}}$ at both temperatures by approximately 1-log, however pressure only affected $K_M$ at 40 °C but not at 80 °C. Increased $K_M$ indicates that the enzyme substrate complex formation is being hindered at 40 °C but not at 80 °C. The $V_{\text{max}}$ was lower at 80 °C and low pressure than at 40 °C and 200 MPa which may be attributed to lipase inactivation at high temperatures and low pressures.

The application of high hydrostatic pressure (HHP) on the apparent kinetics of immobilized and free lipase (Candida antarctica lipase B) in a biphasic ionic liquid (IL)-alcohol system was investigated. ILs have become an attractive alternative media because they can enhance stability, enantioselectivity, product yield, and reaction rate of enzyme reactions. Although the application of IL and HHP to enzyme catalysis has been previously explored separately, this study is the first to explore the combination of these technologies. Production of isoamyl acetate was up to 10-fold higher with free lipase versus immobilized lipase after 3 h at 300 MPa and 80 °C. Rate of catalysis by free lipase also increased up to 15 and 25-fold at 500 MPa versus at 0.1 MPa at 40 or 80 °C, respectively. Pressure affected the activation energy ($E_a$) of immobilized lipase but not free lipase (43.4 ± 3.1 and 55.4 ± 0.1 kJ mol$^{-1}$ at 0.1 and 300 MPa respectively). Temperature had no effect on activation volume ($\Delta V^\ddagger$) which was -16.1 ± 1.5 and -16.7 ± 1.4 cm$^3$ mol$^{-1}$ at 40 and 80 °C respectively. It was also observed that after treatment at high pressure, the free lipase is temporarily suspended in a semi-solid IL phase. This “temporary immobilization” has not been previously described, and may be useful in aiding the separation of phases between reaction cycles.
This study has demonstrated how high pressure can induce activation and stabilization of lipase in a variety of solvents. However, there is a significant lack of data describing the continuous \textit{in situ} conformational changes induced by combinations of temperature and pressure that may be causing activation and/or stabilization. Fluorescence spectroscopy has been conducted \textit{in situ} at pressures up to 500 MPa and up to 80 °C. Results indicate immediate and profound changes in tryptophan fluorescence as indicated by changes in the intensity at 350 nm. Although these results are qualitative, they do provide a valuable insight into the conditions which trigger conformational changes.
CHAPTER 1
INTRODUCTION

History

In June of 2007, the scientific community celebrated the 30th anniversary of the discovery of volcanic hot vents at the Galapagos Rift at the bottom of the Pacific Ocean. The most notable finding was not the vent itself, but the abundance of entire ecosystems at these extreme conditions (up to 120 MPa, 2–100 °C, absence of sunlight, and scarce supply of organic nutrients) which led to a fundamental change in our understanding of life on Earth. While the presence of barophilic (also termed piezophilic) bacteria was first hypothesized in 1949 [1] and later discovered in various trenches during the Galathea Deep-Sea Expedition of 1950-1952 [2], this was the first discovery of multi-cellular life in the deep sea. These discoveries spawned a global quest for life from extreme conditions. This search has resulted in the discovery of over 550 species thriving at various extreme pressures and temperatures and new species continue to be discovered at a rate of about two per month in environments as extreme as 470 °C [3]. Discoveries of both thermostable and piezostable microorganisms and their enzymes have enabled the field of high pressure enzyme modification to progress dramatically by uncovering several high pressure-induced enzyme applications [4] and modifications.

Since the discovery of microorganisms in deep-sea sediments [5, 6] in the 1880’s, high pressure (HP) and its application to bioscience was only briefly demonstrated in the early 1900’s [7]. While HP was effective at inactivating various deleterious enzymes [8-14], its effect on enzyme stabilization and activation was explored less extensively [4, 15-19]. Enzymes from mesophilic and extremeophilic microorganisms were stabilized
by HP [20, 21]. Extremophiles are defined as organisms that have evolved to exist in extreme environments and fall into at least 15 classes of which the most common are thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles, among others [22]. Within the context of this review, thermophilic organisms will be sub-categorized into moderate thermophiles (45-65 °C), thermophiles (65-85 °C), hyperthermophiles (85-100 °C), and extreme thermophiles (>100 °C).

Extremely heat-tolerant (stable up to 150 °C) enzymes have potential application in organic media [22-24]. However, the boiling point of most organic solvents is well below 100 °C. As pressure is raised, the boiling point of organic solvents increases allowing studies above conventional temperatures at 0.1 MPa [25]. The ability to operate with organic or volatile solvents at temperatures above their conventional boiling point is an important yet often overlooked advantage of HP.

The cost of high pressure processing (HPP) 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 [26] with 80% of their equipment installed since 2000. HPP food became a two billion dollar global market and is expected to comprise 450 million pounds/year in 2008 [27]. As demand for HPP equipment grows, innovation is expected to continue to reduce capital and operating costs [26]. Although HPP of bulk foods is currently more widespread, the much higher profit-margin sector of enzyme catalyzed synthesis of high added-value products such as pharmacological peptides, antibiotics, carbohydrates, and food flavors has greater potential for adopting HPP.
The effects of HP on proteins and enzyme inactivation was discussed and reviewed over the past 20 years [16, 18, 19, 28-31]. Theories of pressure effects on enzymes and the activation volumes of several enzymes were compiled in the early 1980’s [32]. However, the application of HP to enhance enzyme catalysis has not been extensively explored or comprehensively reviewed.

Overview of Current Enhancement Techniques/Technologies

The major drawback to the extensive use of many enzymes compared to chemical catalysts is their relatively low stability in their native state and their often prohibitive cost [33]. There is a great interest in developing competitive biocatalysts for industrial applications by improvement of their activity, stability, and re-usage capacity. Such improvements have been approached by chemical, physical, or genetic modification of the native enzyme. The effects of immobilization on activity, stability, and even selectivity of some enzymes have been well documented [34-36]. For example, immobilization of lipase can improve activity by shifting the equilibrium between open and closed form towards the open, more active form [35]. Also, chymotrypsin can be stabilized by a factor of 60,000 by immobilization [37]. Furthermore, the enantiomeric ratio of lipase products may be changed from 1 to almost 100 by using different immobilization preparations [35]. Furthermore, modification of the reaction environment was explored using alternative solvents including organic solvents such as hexane [38-41], solvent-free systems [42-48], dense or supercritical gases such as supercritical carbon dioxide (SC-CO₂) [49-51], and more recently ionic liquids [52-54]. For example, stability and selectivity of lipase in ionic liquid increased 2.7-fold and by 2%, respectively, when compared to in hexane [54]. Likewise, lipase activity dramatically increased in SC-CO₂ with a negative activation volume of 1340 cm³ mol⁻¹ [55].
Enzymes suitable for use in industrial biocatalysis may require application of a combination of these improvement methods.

**Mechanisms of Pressure-Induced Stabilization**

Although the mechanisms of HP-induced stabilization has yet to be fully elucidated, it appears that intramolecular interactions, hydration of charged groups, disruption of bound water, and stabilization of hydrogen bonds may all play a role. The pressure effects on intra- and intermolecular interactions within proteins were reviewed in depth [56].

According to Mozhaev et al. [57], a possible explanation of pressure-induced stabilization of enzymes against thermal inactivation rests in opposing effects of pressure and temperature on the ability of protein functional groups to interact with water. The promotion of charged species in aqueous medium is favored by a high-pressure environment, because the electrostriction of water around the charges decreases the molar volume of water [58]. This means that an increase in pressure will weaken the electrostatic or coulombic interactions. Hydration of charged groups by water molecules is strengthened by pressure and weakened at high temperature [18, 28]. Evidence of this phenomenon was presented by Kitchen et al. [59] who completed a molecular dynamics simulation of bovine pancreatic trypsin inhibitor (BPTI) in solution at HP and found large pressure-induced changes in the structure of the hydration shell, the shell appeared more ordered at HP, and that HP-induced greatest ordering for nonpolar surface groups. Enzymatic catalysis in organic media also depends on enzyme hydration state which is greatly influenced by pressure [60]. Mozhaev et al. [57] hypothesized that, at an initial step of thermal inactivation, a protein loses a number of essential water molecules, and this loss may give rise to structural rearrangements in a
protein. HP may hamper this process by favoring hydration of both charged and nonpolar groups. In other words, application of high hydrostatic pressure fortifies the protein hydration shell thus preventing thermal inactivation.

Hydrogen bonding may also be slightly strengthened by an increase in pressure, because of, according to Kunugi [58], the decrease in the inter-atomic distance leading to a smaller molecular size. This phenomenon was initially based on the negative activation volumes associated with hydrogen bond formation in organic compounds such as formic acid, ε-caprolactam, phenol, and poly-L-lysine [61]. Later, the lengths of existing hydrogen bonds within proteins have been observed to be shorter under HP as first detected by nuclear magnetic resonance (NMR) [62, 63] and then by low-frequency Raman spectroscopy [64].

Hydrophobic interactions also greatly affect enzymatic conformation. The relationship between HP and temperature effects on hydrophobic interactions has yet to be fully understood [56]. It was suggested that hydrophobic interactions are strengthened by HP because the hydration of non-polar surface molecules is not favored due to the corresponding volume increases [30, 65]. Evidence supporting this hypothesis was provided by Hei and Clark [66] by demonstrating how the stability of several glyceraldehydes-3-phosphate dehydrogenases (GAPDHs) with varying hydrophobicity of their “s-loops” (structural motifs consisting of residues 178 to 201 which are an important determinant of GAPDH thermal stability) is correlated to pressure-induced stabilization. Further evidence was provided by Mei et al. [67] who studied the role of hydrophobic residues on pressure-induced stabilization. Through site-directed mutagenesis, Mei et al. [67] modified the hydrophobicity of azurin and
pressure-induced stabilization increased with increasing hydrophobicity. However, this was in disagreement with Mozhaev et al. [18] who said “it is generally accepted that formation of hydrophobic contacts proceeds with positive $\Delta V^\neq$ and is therefore disfavored by pressure” and cited the formation of hydrophobic interactions when transferring methane from a non-polar solvent to water, the micelle formation by surfactants, and a dimerization of carboxylic acid [68, 69] which all resulted in a positive $\Delta V^\neq$. However the dimerization of formic, acetic, propionic, and n-butyric acid all had negative $\Delta V^\neq$ [69].

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 more recently with regards to enzyme conformation within defined pressure and temperature regions [17-19, 28]. This defined region must be determined experimentally as the complexity of using an enzyme catalyst prevents accurate modeling without pre-determination of enzyme stability and activity at various pressure-temperature combinations. The relationship between pressure and temperature effects on enzyme conformation often results in an elliptical pressure versus temperature diagram as shown in Fig. 1 with native and denatured protein regions inside and outside of the ellipse, respectively, separated by a zone of reversible denaturation with the contour line representing where the equilibrium concentrations of native and denatured forms of the enzyme are equal.

A comprehensive review of pressure-temperature phase diagrams of protein unfolding was compiled by Smeller [70]. Increases in temperature and pressure can
have both, stabilizing or denaturing effects on enzymes depending on the initial conditions, as represented by points A, B, C, and D in the elliptical diagram and the magnitude of the increase. For example, moderately increasing temperature and pressure from point A stabilizes the enzyme. However, in point D, increases in both, temperature or pressure destabilizes the enzyme. In the case of point B an increase in pressure denatures the enzyme but an increase in temperature stabilizes it and vice-versa for point C. Such diagrams were first shown by Hawley [71] and later reviewed and discussed by several others [16, 17, 70, 72]. However, they are typically representations of the rate of denaturation which doesn’t always directly correlate to inactivation. These diagrams have been used to describe pressure stability of chymotrypsin [71], polyphenoloxidase [73, 74], pectin methylesterase [75-77], myrosinase [78], naringinase [79], β-glucanase [80], α and β-amylase [81, 82], and glucoamylase [83].

**Mechanisms of Pressure Induced Activation**

Explanations for pressure-induced changes in the rate of enzyme-catalyzed reactions can be classified into: (1) direct 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 physical properties (e.g. pH, density, viscosity, phase) that affect enzyme structure or the rate-limiting step.

Early studies suggested that the activities of monomeric enzymes activity was stimulated, while the activities of multimeric enzymes were inhibited by application of high hydrostatic pressure [56]. However, at least 15 dimeric and tetrameric enzymes have been reported to be activated by pressure [32].
It is understood that pressure effects on reaction rates adhere in most cases to Le Châtelier’s principle: that elevated pressures favor changes that reduce a systems’ 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 rate and equilibrium can be estimated [28] using the Eyring equation (Eq. 1-1) [57],

\[
\left( \frac{\partial \ln k}{\partial p} \right)_T = -\frac{\Delta V^\neq}{RT}
\]

(1-1)

where \( p \) is the pressure, \( T \) is the absolute temperature, \( R \) is the ideal gas constant, \( \Delta V^\neq \) is the activation volume that represents the dependence of the reaction rate with pressure, and \( k \) is the rate constant. After integration and rearrangement, Eq. (1) can be rewritten as Eq. (1-2).

\[
\ln k_0 = -\frac{\Delta V^\neq}{RT} \cdot P + \ln k_{P_0}
\]

(1-2)

where \( k_0 \) is the rate constant at a reference pressure \( P_0 \). Thus, any rate with a negative volume change will be shifted toward the more compact state by increasing pressure and any reaction with a positive activation volume will be slowed.

Compared to chemical catalysis, it is more difficult to give a precise physical meaning to \( \Delta V^\neq \) for enzyme reactions because pressure affects several factors including: enzyme conformation, enzyme solvation (interaction with surrounding medium, other proteins, water, ions, etc.), chemical equilibrium, and changes in immobilization support or immobilization bonding. Depending on the reaction or
conformational changes of the enzyme (which are dependent on the temperature-pressure range), activation volume can be positive, negative, or negligible. Typically $\Delta V^f$ ranges from $-70 \text{ cm}^{-3} \text{ mol}^{-1}$ to $+60 \text{ cm}^{-3} \text{ mol}^{-1}$, with most having a magnitude of less than $30 \text{ cm}^{-3} \text{ mol}^{-1}$ [28].

Separating the effects of HP on the rate constant from those on enzyme conformation and thus on the availability of active enzyme at elevated pressures is difficult because they both occur simultaneously affecting the rate of catalysis. The simple one-substrate Michaelis-Menten mechanism and equation are shown in equations 1-3 and 1-4.

$$E_n + S \overset{k_1}{\rightleftharpoons} E_nS \overset{k_2}{\rightarrow} E + P$$

(1-3)

$$v = \frac{V_{max} [S]}{K_M + [S]} = \frac{k_2[E_T][S]}{K_M + [S]}$$

(1-4)

where $E_T$ is the total enzyme concentration ($[E]+[ES]$), $[S]$ is the substrate concentration, $v$ is the rate of reaction, and $k_1$, $k_2$, and $k_{-1}$ represent rate constants and $K_M$ is Michaelis-Menten constant. Large increases in pressure, temperature, or addition of a denaturing agent causes the native enzyme ($E_n$) to first become reversibly ($E_r$) and then irreversibly ($E_d$) inactivated as described in Eq. (1-5). However, moderately increasing pressure can produce a more active enzyme ($E_{act}$) as shown in Eq. (1-6) which accounts for pressure induced activation.

$$E_n \leftrightarrow E_r \rightarrow E_d$$

(1-5)
The rate constant is also affected by temperature and generally expressed by the Arrhenius equation (Eq. 1-7)

$$\ln k = - \frac{E_a}{R} \cdot \frac{1}{T} + \ln k_{T_0}$$  \hspace{1cm} (1-7)

where $E_a$ is the activation energy and $k_{T_0}$ is the rate constant at a reference temperature $T_0$. Both temperature and pressure may be complementary to activation at a range of temperatures and pressures; however, it can also hinder the reaction rate by inactivating the enzyme. The combined effect of pressure and temperature is currently impossible to predict without some preliminary data,

In the past 25 years, enzyme catalysis in organic media (mainly n-hexane) was the focus of many studies and reviews [24, 39, 40, 58-65]. The pressure dependence of the solubility parameter and molar volume of organic solvents was recently described at pressures up to 300 MPa at 30 °C [66]. Pressure monotonically increased the solubility parameter (~20%) and decreased the molar volume (16%) of many of the commonly used organic solvents. Use of non-polar solvents results in better esterification (i.e. higher conversion rates, increased stability, etc.) as they preserved the catalytic activity without disturbing the aqueous mono-layer of the enzyme [67]. The pressure effect on increasing polarity of organic (non-polar) solvents may hinder esterification as product formation is favored in less polar solvents. However, the flexibility of some enzymes is increased with increasing polarity of the organic solvent [68]. Therefore, pressure may increase conformational flexibility, thus improving reaction rates as conformational
flexibility is required for activation of enzymes that show interfacial activation in the presence of substrate.

Activity of lipase [41, 69], thermolysin [70], esterase (from *Bacillus licheniformis*) [71], chymotrypsin [72, 73], xanthine oxidase [74, 75], horseradish peroxidase [76], polyphenol oxidase [76], subtilisin [73], protease (from *Bacillus subtilis*) [73], and alcohol dehydrogenase [77] are active in organic or non-aqueous media. Enzyme activity in organic solvents can be correlated with the solvent polarity [40, 41, 78]. However it has also been reported that activity might be affected by the solvent without correlation to the polarity [79, 80]. It was suggested that enzymes that are active in organic solvents retain their native structure upon transfer from water to organic solvents [73]. This is evidenced by the phenomenon of pH memory discussed by Klibanov [76] which suggests the presence of trapped active sites in organic solvents [64, 73] and the observation that the addition of strong acids to lipase in hexane does not appreciably inactivate the enzyme [81]. 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 [64, 73]. In contrast, fully dry enzymes are inactive and enzymes in organic solvents with high amounts of water show denaturation [64, 73]. Therefore, it is reasonable to hypothesize that pressure-enhanced activity of enzymes in low or non-aqueous solvents may be partially attributed to the pressure-induced hydration of charged groups.

Solvent compression at HP increases substrate molar concentration producing an increase in reaction rate. Solvent compression also results in increased viscosity, which in results in a decrease in reaction rate particularly in heterogeneous systems. Because
most enzymes utilized in organic media are immobilized (due to limited solubility), viscosity effects may be significant unless adequate turbulence is provided.

The objective of this paper is to compile and critically present reports of HP enzyme enhancement as an effective reaction parameter with potential for greater utilization in enzyme catalysis.

**Enzymes Enhanced by High Pressure**

Discussion of the effects of pressure on enzyme stability and activity is organized by enzyme commission (EC) number.
Oxioreductases (EC. 1)

Dehydrogenase (EC 1.1.1.1).

Dehydrogenases and hydrogenases are widely distributed in nature and have been found in many microorganisms, plants, and animals and are the key enzyme involved in the metabolism of H\textsubscript{2}. Studies in the 1960s with malic dehydrogenase from Bacillus stearothermophilus had no activity at 101 °C from 0.1 to 70 MPa. However, activity was induced above 70 MPa with optimal activity at 130 MPa at 101 °C [85]. This relative activation was attributed to pressure counteracting the increased volume associated with enzyme denaturation. HP effects on the oxidation of benzyl alcohol by yeast alcohol dehydrogenase were measured by substrate capture. Moderate pressure increased the rate of capture of benzyl alcohol 4-fold at 150 MPa and was attributed to activating the hydride transfer step [84].

Pressures up to 75.9 MPa stabilized two glutamate dehydrogenases (GDH) from the hypothermophile Pyrococcus furiosus and a recombinant GDH mutant [82]. The stabilizing effects of pressure increased with temperature reaching 36-fold for recombinant GDH at 105 °C and 75.9 MPa. Likewise, GDH from the liver of the Antarctic fish Chaeonocephalus aceratus remained active up to 140 MPa and had slightly increased activity from 2 to 100 MPa compared to 0.1 MPa, although reaction rate decreased with pressure above 100 MPa [132]. Fish GDH was rapidly inactivated above 200 MPa while bovine GDH was inactivated slowly in the range of 220-290 MPa [132, 133].

Pressure inhibited the tetrameric mesophilic yeast alcohol dehydrogenase (YADH), whereas the thermostable Thermoanaerobium brockii alcohol dehydrogenase
(TBADH) was activated by pressure up to 100 MPa [83]. These results supported the hypothesis that HP can stabilize enzymes from thermophiles and that pressure stabilization may be related to increased hydropobicity of surface groups typically associated with thermophilic enzymes as discussed earlier in section 1.3. Furthermore, differences in genetic sequences (Ala-180 and His-229) of dehydrogenases have been discovered between two dehydrogenases from the same organism (Moritella sp. strain 2D2) found in vastly different pressure and temperature environments [134, 135] and provided further evidence that pressure-induced stabilization or activation may be related to a specific genetic sequence or set of sequences as discussed in section 1.3.

**Hydrogenase (EC 1.1.1.2)**

The relationship between thermophilicity and pressure stabilization was explored using partially purified hydrogenases from a mesophile, *Methanococcus maripaludis*; a moderate-thermophile, *Methanococcus thermolithotrophicus*; a hyper–thermophile, *Methanococcus igneus*; and the extreme-thermophile deep-sea methanogen, *Methanococcus jannashii*. HP from 1.0 to 50.7 MPa increased the half-life of the hydrogenase from *M. jannashii* and *M. ignius* at 90 °C 4.8- and 4.5-fold, respectively, while pressure decreased the half-life of *M. thermolithotrophicus* and *M. maripaludis* [86]. Hydrogenase activity from *M. jannashii* more than tripled by an increase from 0.75 to 26 MPa at 86 °C with a corresponding $\Delta V^\ddagger$ equal to $-140 \pm 32 \text{ cm}^3 \text{ mol}^{-1}$ [87]. These results further supported the hypothesis that barophilicity increases with thermophilicity.

**Peroxidase (1.11.1.1-16)**

Peroxidases (POD) constitute a group of thermostable heme proteins that oxidize substrates (monophenols, diphenols, etc.) while utilizing $\text{H}_2\text{O}_2$ [136]. Although POD often has a deleterious role in food quality, it can also be used as a catalyst in many
industrial applications, including wastewater treatment, fine chemical biosynthesis, and even paper pulp treatment [137]. While POD is widely used, its main limitations are enzymatic stability and low production yield.

High pressure treatment (HPT) increased activity of strawberry POD by 13% and 1% at 400 MPa when applied for 5 and 10 min, respectively [88]. Similarly, crude extract of mate tea leaf POD increased activity 25% after exposure to compressed CO₂ at 30 °C and 7 MPa [136] and was attributed to the opposite effects of pressure and temperature on the ability of the protein functional groups to interact with water. Carrot and apple POD were also quite resistant to pressurization below 900 MPa for 1 min, and activation was observed for treatments at 300-500 MPa [95].

**Lipoxigenase (EC. 1.13.11.12)**

Lipoxygenase (LOX) is a non-heme iron-containing dioxygenase that is found throughout the plant kingdom, especially in legumes [89]. It causes chlorophyll destruction and off-flavor development in frozen vegetables and is, therefore, a target for inactivation. Green pea LOX demonstrated resistance to thermal inactivation above 60 °C under 200 MPa [89]. Likewise, LOX from soybean activity increased 120% after treatment at 200 MPa and 55 °C for 15 min in 30% sucrose solution compared to residual activity at 0.1 MPa [90]. Although LOX is generally considered a deleterious enzyme in foods and a target for inactivation, pressure-induced stabilization or activation provide further evidence toward the role of HP in enzyme enhancement.

**Polyphenoloxidase (EC 1.14.18.1)**

Polyphenoloxidases (PPO) are a group of copper proteins that catalyze the oxidation of phenolics to quinones that produce brown pigments. They are widely distributed in plants, animals, and microorganisms. The oxidation of phenolic substrates
by PPO is a major cause of browning in foods during ripening, handling, storage, and processing. It is estimated that over 50% of losses in fruits occur as a result of browning [138]. However, PPO is vital for the desirable color and flavor generation characteristic of many plant based foods.

Mushroom PPO activity increased 140% after treatment at 400 MPa for 10 min compared to untreated sample; and even after 10 min at 800 MPa about 60% residual activity remained [93]. The enhancement in activity was hypothesized to be from pressure-induced changes in the interactions with other constituents in the extract or from the release of membrane-bound enzymes. Similarly, PPO from pears was activated at 400 MPa with a maximum 5-fold increase activity at 600 MPa and 20 °C for 6 h [139]. Unlike the mushroom extract, PPO from pear showed pressure-induced activation which could not be explained by interactions with other constituents as the PPO was extracted and purified to homogeneity. It was suggested that such activation is a result of a limited conformational change in the enzyme, release of the enzyme from an enzyme-inhibitor complex, or activation via limited proteolysis such as zymogen activation [140]. Asaka and others [140] concluded that PPO of La France pear fruit in its latent form is activated by a limited conformation change; however, such a mechanism is not known.

Victoria grape must PPO experienced a synergistic effect between pressure and temperature for inactivation at 20 to 60 °C and 200 to 800 MPa and antagonistic effects at above 60 °C and below 200 MPa [96]. Above 600 MPa, a temperature increase from 50 to 80 °C was needed to accelerate PPO inactivation by a factor of 10 [141]. Below 300 MPa, the inactivation rate constant decreased with increasing pressure which was
ascribed to the antagonistic effect of pressure and temperature [141]. A similar antagonistic effect was observed for avocado PPO at high temperature (T ≥ 62.5 °C) and low pressure (P ≤ 250 MPa) where a pressure increase caused a decrease in inactivation rate [91]. It was also shown that avocado PPO exhibits HP stability (900 MPa for 270 min produced only 1 log-reduction of activity) at room temperature [91]. Red raspberry PPO was activated at 400 and 800 MPa by 15 and 8%, respectively [88]. Likewise, PPO cell-free extracts from carrots and apples showed a dramatic increase in enzyme activity at pH 4.5 and 5.4 after pressurization at 100 to 300 MPa for 1 min [95]. The activation effects, observed for moderate pressure treatments was attributed to reversible configuration and or conformation changes on enzyme and/or substrate molecules [95].

Onion extract PPO activity increased up to 500 MPa with maximal activity of 142% compared to untreated enzyme [92]. The mechanism of this pressure-induced enhancement was not explored but was suggested to be due to pressure-induced protein conformational changes or separation of a part of the protein molecule and subsequent liberation of a second active site. However, there was no indications of major changes in native and pressure treated onion PPO when analyzed using SDS-PAGE [92].

**Transferases (EC 2)**

**Polymerase (2.7.7.7)**

With the advent of PCR (polymerase chain reaction), DNA polymerases have become vital in molecular biology [142]. DNA polymerase is the most heat labile enzyme from the *Pyrococcus* species [143]. DNA polymerases derived from three thermophilic microorganisms, *Pyrococcus* strain ES4, *Pyrococcus furiosus*, and
*Thermus aquaticus* were stabilized by HP at 111 °C, 107 °C, and 100 °C, respectively [21]. Half-lives of the three DNA polymerases increased from 5.0, 6.9, and 5.2 min, respectively, at 3 MPa to 12, 36, and 13 min, respectively at 45 MPa [21]. Pressure above 45 MPa did not significantly increase the half-life of *P. furiosus* DNA polymerase, whereas pressure increase up to 89 MPa did further increase the half-life of DNA polymerase from *Pyrococcus* strain ES4 and *T. aquaticus* [21]. Pressure-induced stabilization was attributed to pressure effects on thermal unfolding or retardation of unimolecular inactivation of the unfolded state, and differences between strains is attributed to differences in the amino acid sequences, which correlate to pressure-induced stabilization. While application of HP for conducting a PCR assay may not be currently suitable with today’s technology, any method for stabilizing an enzyme that is as widely used as polymerase is worthy of further investigation.

**Hydrolases (EC 3)**

**Pectin methylesterase (EC 3.1.1.11)**

Pectin methylesterase (PME) catalyzes the demethylesterification (or demethoxylation) of pectin releasing methanol and protons (creating negatively charged carboxyl groups) [101, 144] which reduces viscosity and forms low-methoxyl pectin precipitates resulting in cloud loss of fruit and vegetable juices. This enzyme has been subject to recent reviews which summarize PME genetic sequencing, elucidation of three-dimensional structure, the multiple roles of PME in plants, and the existence of multiple isozymes which differ in their thermal and pressure stability [102].

The rate of recombinant *Aspergillus aculeatus* PME enzymatic de-esterification was optimal at 200-300 MPa combined with 45-55 °C [99]. Similarly, the highest rate of PME-catalyzed pectin de-esterification was at 200-300 MPa at 50-55 °C [98]. This
increase in reaction rate of PME was explained using Le Chatelier’s principle which may be applicable for de-esterification reactions because charged groups are formed, and solvation of these charged groups is accompanied by reduced volume resulting from electrostriction; i.e. the compact alignment of water dipoles owing to the columbic field of the charged groups [16]. However, Le Chatelier’s principle is only applicable as an all-encompassing explanation if pressure does not significantly affect PME stability. In exploration of this caveat to the Le Chatelier’s principle explanation, Fourier transform IR spectroscopy was used to reveal the pressure stability of β-helices of Aspergillus aculeatus PME [97]. No significant changes in spectra could be observed up to 1 GPa. At pressures above 1 GPa, unfolding takes place as indicated by broadening of the deconvoluted amide I’ band from approximately 1638 cm\(^{-1}\) towards approximately 1635 cm\(^{-1}\) [97]. Pressure stabilization was explained by pressure-induced suppressive effect on the aggregation of the partially unfolded protein due to the counteracting hydrophobic forces to a certain extent and by the substantial amount of protein in the β-helix conformation [97].

Tomato juice PME was activated by pressure above 300 MPa at all temperatures, and maximal at 300 MPa and 50 °C which was almost 1.7 times the control at 0.1 MPa [103]. This activation was attributed to reversible conformational changes or the enzyme and/or substrate molecules. In a related study, PME from tomato was quickly inactivated at 0.1 MPa and 70 °C but the inactivation rate constant dramatically decreased as soon as pressure was raised reaching a minimum between 300 and 600 MPa [94].
Extracted and purified carrot PME showed notable pressure resistance (up to 600 MPa) to inactivation in combination with increased catalytic activity with increasing pressure [101]. The most pronounced activity of carrot PME was observed at 50 °C and 500 MPa. Similarly, PME activity in shredded carrots (*in situ*) was most pronounced at 50 °C and 200-400 MPa and 100-400 MPa at 60 °C in intact carrots [101]. Similar results were found with extracted and purified carrot PME at lower pressures (300 MPa) and higher temperatures (> 50 °C) where an antagonistic effect of pressure and heat was observed [102]. At 60 °C, the carrot PME inactivation rate was reduced by greater than 75% at 300 MPa versus 100 MPa [102]. Since similar results between carrot PME in shredded carrots and with extracted and purified PME indicate that pressure induced activation is not related to pressure effects on the substrate (pectin or pectate).

Pressure-treated green peppers showed increased PME activity after treatment between 100 and 200 MPa for 10 or 20 min compared to the native control [145] and up to 500 MPa at room temperature [146].

Extracted and purified PME from bananas exhibited the antagonistic effect of pressure and temperature in the low pressure (≤ 300 MPa) and high-temperature domain (T ≥ 64 °C). Increased pressure resulted in a decrease of the observed inactivation rate constant [97]. Inactivation rates of orange PME were also slower at 80 °C and 900 MPa compared to atmospheric pressure and was more pronounced in the presence of Ca^{2+} ions (1 M) but less pronounced for the inactivation in an acid medium (pH 3.7) [100].

While PME is often a target for inactivation in cloudy juices, it is vital for reducing viscosity of high pectin mixtures and the clarification of cloudless juices such as apple
juice. It appears that HHP may be utilized to stabilize PME where needed while inactivating various other undesirable enzymes during a combined pressure-heat treatment.

**Lipase (EC 3.1.1.3)**

Lipases (triacylglycerol acylhydrolases) occur widely in nature where they catalyze the hydrolysis of esters from glycerol and long-chain fatty acids releasing alcohol and acid moieties. Since it became accepted that lipases remain enzymatically active in organic solvents [65] and react in the opposite direction (synthesis of esters), their application grew and have been the subject of many reviews and studies [49, 147-156]. Lipases now constitute the most important group of biocatalysts for the synthesis of biopolymers and biodiesel, resolution of racemic mixtures, transesterifications, regioselective acylation of glycols and menthols, synthesis of peptides, and the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds [153]. Because of their importance to a variety of industries, lipases have been explored and documented more than any other enzyme at ambient pressure and at HP. Therefore, lipase is discussed in this review to a greater depth than any other enzyme.

Lipase stability and activity was examined at HP in compressed or supercritical fluids/gases including carbon dioxide [49, 107-109, 157-167], propane [51, 109, 110, 167], butane [51, 110], sulfur hexafluoride (SCSF₅) [51], and mixtures of butane and propane [51]. *Candida Antarctica* lipase B (CALB) was effective at synthesizing isoamyl acetate in SC-CO₂ at higher initial reaction rates when compared to n-hexane while maintaining activity from 8 to 30 MPa [161]. These results were explained as being due to improved diffusivity of the reactants in the medium. CALB was also successfully used as a catalyst to synthesize butyl butyrate in SC-CO₂ [108]. All supercritical conditions
assayed enhanced activity 84-fold with respect to synthesis in organic solvents while maintaining stability showing a 360 cycle half-life. Best results were achieved at 60 °C and 8 MPa and were explained as improved micro-environments around the enzyme [108].

The apparent kinetics of immobilized CALB synthesis of isoamyl acetate at HHP in hexane has also been examined. Activity of CALB 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. $\Delta V^\ddagger$ appeared negative between ambient pressure and 200 MPa in contrast to a positive $\Delta V^\ddagger$ between 300 and 600 MPa. Besides increasing activity, HHP also reduced thermal inactivation of CALB by up to 152% after 4 h at 80 °C and 400 MPa when compared to incubations at low pressure [168]. Lipase activity at 63.5 and 80 °C at 400 MPa was greater (from about 20 to 96% increase) than at low pressure [168].

**Rhizomucor miehei** lipase was used in the esterification of lauryl oleate from oleic acid and 1-dodecanol in a batch stirred reactor using dense CO$_2$ as a reaction medium where the catalytic efficiency increased up to 10 MPa and was attributed to either substrate-solvent clustering, stabilization effect of SC-CO$_2$ treatment, or to the stabilization of lipase in the “open” form by hydrophobic interactions [104]. HHP in an aqueous medium has also been shown to improve lipase from **Rhizomucor miehei**. At denaturing temperatures (50, 55, and 60 °C), application of 50 to 350 MPa protected the lipase. This protective effect increased with temperature [106]. Similarly, the protective effect of pressure against thermal inactivation (50 and 55 °C) was observed for
*Rhizomucor miehei* lipase at 50 – 450 MPa [105]. Conformational studies using fluorescence spectroscopy suggested that the conformational changes induced by pressure were different from those induced by temperature [169]. Noel and Combes [169] also concluded that *Rhizomucor miehei* lipase has a high stability under pressure as it represents a slight change in volume (0.2%) in comparison to the initial volume of the native protein. Conversely, Osorio and others [170] found for lipase from *Thermomyces lanuginose* increasing pressure improved selectivity while it linearly decreased stability.

Catalytic efficiency of *Pseudomonas cepacea* lipase transesterification of 1-phenylethanol and vinyl acetate in SC-CO$_2$ increased up to 15 MPa [55] and was more thermostable in supercritical propane than in water (optimum temperature increased from 36.8 °C in water to 49.8 °C in propane) [167]. This was hypothesized to be a consequence of protein structural and conformational rigidity in propane. In the same study lipases from *Pseudomonas fluorescences*, *Rhizopus javanicus*, *Rhizopus niveus*, and *porcine pancreas* were stable in SC-CO$_2$ and near-critical propane as activity remained constant after 24 h at 30 MPa and 40 °C. Recently, activity of lipase from porcine pancreas, *Candida antarctica*, *Aspergillus oryzae*, *Candida cylindracea*, *Penicillium roqueforti*, *Aspergillus niger*, *Rhizopus arrhizums*, *Mucor miehei*, and *Pseudomonas cepacia* was increased after reacting at 40 °C and 15 MPa in SC-CO$_2$ [171]. The most remarkable increase in activity was from *Rhizopus arrhizums* lipase where activity increased 50 fold [171].

Lipase from *Candida cylindracea* used for the esterification between n-valeric and citronellol was shown to have a reaction rate dependence on pressure [165] which
increased significantly until reaching a maximum at 7.55 MPa near the critical point of SC-CO₂ [165]. Increased activity was explained by interactions between carbon dioxide and enzyme molecules inducing drastic conformational changes of the enzyme, causing active sites to emerge and catalyze the stereoselective synthesis [165].

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 [172]. 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 [173]. This “lid” is shifted aside thus exposing the active site and generating the “active” form of lipase. This mechanism was suggested [104] for lipase (lipozyme RM-IM) activation at pressure close to 10 MPa in CO₂. Knez and others [104] explained in such a condition the “lid” is moved away (rolls back) and the hydrophobic CO₂ molecules stick out around the active site, following the opening of the lid, unblocking the entrance of the tunnel. Uppenberg and others [172] suggested that the α-helix (α5) can be stabilized by hydrophobic molecules at the entrance of the active site pocket.

Recent studies conducted in aqueous media have concluded that in contrast to most lipases, CALB may not have a “lid” blocking access to the active site and shows no interfacial activation [68] as compared to Candida antarctica lipase A (CALA) which does possess the characteristic “lid” structure and shows interfacial activation [174]. 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 [68]. Flexibility limited to these five elements vital to catalysis may offer possible explanation for relatively HP stability, while still allowing modification of activity. Since lipases are widely used in high value product (fine flavors, pharmaceuticals, etc.) biosynthesis, the use of HP technologies may be uniquely suitable due to cost effectiveness associated with these products.

**Acetylcholinesterase (EC. 3.1.1.7)**

Acetylcholinesterase is indispensable in synaptic transmission by catalyzing hydrolysis of acetylcholine and plays a role in stress response, degenerative diseases, and neural development in humans [175]. Acetylcholinesterase 7.4S was more active at pressures up to approximately 14 MPa when compared to 0.1 MPa [112]. Pressure induced stability appeared to be related to the 7.4S enzyme having a large volume expansion accompanying the activity loss apparently retarded by application of moderate to low pressure. This enzyme is the only pressure-enhanced enzyme native to human physiology neural synaptic system. Acetylcholinesterase is widely used in various biosensors mainly for the detection of pesticides but also for detection of metal cat ions, inorganic metals, as well as other organic compounds [176]. Therefore it may be a suitable candidate for a pressure stable biosensor used in HP environments such as deep-sea exploration and deep underground oil, natural gas, and water drilling.

**Polygalacturonase (EC 3.2.1.15)**

Polygalacturonase (PG) catalyzes the α-1,4-glycosidic bonds of the product of PME catalyzed pectin de-esterification. This depolymerization leads to a drastic decrease in viscosity of various fruit and vegetable juices. Unlike PME, increases in pressure and temperature can accelerate the inactivation rate of PG [94]. However, a
pressure resistant PG fraction from tomato was not affected by prolonged pressurization up to 600 MPa at 50 °C [113]. Furthermore, PG activity increased up to optimal pressure (100-200 MPa) depending on the temperature [177]. Verlent and others [177] found that PG catalyzed hydrolysis of pectin in the presence of tomato PME remains constant with increasing pressure at 50 °C which is in contrast to earlier work by the same group [178, 179] where a shift in optimal temperature to lower values was observed with increasing pressure. The relationship between PG activity with or without PME needs further exploration; however it is clear the HP can be utilized to modulate activities to achieve desired processing conditions such as increasing PG activity to reduce fruit or vegetable juice viscosity.

**α-Amylase (EC. 3.2.1.1)**

α-Amylase catalyzes the hydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units in random order [114] and used mainly to degrade starch to low molecular weight dextrins in the production of sugar syrups [180] as well as for the preparation process of alcoholic beverages such as beer and whisky [114]. Studies from the 1950s described by Laidler [181] identified a “salivary amylase” and a “pancreatic amylase” which both had $\Delta V^\ddagger = -22$ cm$^3$ mol$^{-1}$ and -28cm$^3$ mol$^{-1}$ respectively. More recently, α-amylase from barley malt was stabilized against thermal inactivation up to 200 MPa with maximum substrate cleavage at 152 MPa and 64 °C with 25% higher yield as compared to the maximum at ambient pressure and 59 °C [114]. The optimum conditions are explained by the superposition of pressure induced thermal stabilization of the enzyme and enhanced substrate conversion by temperature. Treatment of α-amylase with SC-CO$_2$ and 0.1%
water at 35 °C and 20 MPa for 1 h resulted in residual activity of 105% compared to prior to treatment [182].

**β-Amylase (EC. 3.2.1.2)**

β-Amylase hydrolyzes the α-1,4-glucosidic linkages of starch from the non-reducing ends. This reaction is utilized in the food and beverage industry for the preparation of fermented foods or maltose production as a precursor for alcoholic beverages. The effects of HP on the flexibility of subgroups within β-amylase have been observed by monitoring intensity changes in the near UV spectrum and with circular dichroism [183]. β-amylase activity from barley malt was strongly affected by HP and temperature treatment [115]. Highest depolymerization of starch rate was found at 100 MPa and 62 °C, yielding 15% more compared to optimum conditions at ambient pressure [115]. The shift in temperature optimum was attributed to the stabilization of the enzyme against thermal inactivation. Like α-amylase, HP may be utilized to improve β-amylase catalysis for use in the production of sugar syrups or alcoholic products.

**β-Glucanase (EC. 3.2.1.2)**

β-Glucanase activity in malt is crucial for the brewing process because of its effect on beer filtration. β-Glucanase depolymerizes β-glucans which increase beer viscosity by thus reducing filterability [184, 185]. β-Glucanases are synthesized during germination but are inactivated at temperatures above 50 °C during the malting and mashing processes. Pressure (400 MPa) significantly stabilized this enzyme against thermal inactivation [116]. However, increasing pressure also decreased activity up to 600 MPa [116]. Counteracting effects of stabilization and reduced activation resulted in maximum conditions at 215 MPa and 55 °C which yielded approximately 2/3 higher degradation of β-glucan after 20 min as compared to the maximum at 0.1 MPa at 45 °C.
No discussion was provided with regards to substrate stabilization, or pressure effects on substrate. It is clear that high hydrostatic pressure along with elevated temperature can be utilized to improve β-glucanase activity for depolymerizing β-glucans.

**Glucoamylase (EC. 3.2.1.3)**

Glucoamylase is a multidomain exo-glycosidase that catalyses the hydrolysis of α-1,4 and α-1,6 glucosidic linkages of starch and related polysaccharides to release β-D-glucose from the non-reducing ends and is used in the production of spirits and glucose syrups from starch [117]. Two isoforms of glucoamylase (GA1 and GA2) from *Aspergillus niger* were studied for their response to pressure temperature treatments. The pressure liable form of glucoamylase (GA2) was inactivated above 850 MPa whereas the stable (GA1) isoform was slow to inactivate up to 1400 MPa at 50 °C [117]. Pressure stabilized the enzymes against thermal inactivation at 400 MPa for GA2 and at 550 MPa for GA1 [117]. Since elevated pressure contributed to delayed enzyme-substrate interaction up to 600 MPa, the maximum production of glucose by maltose cleavage was observed at approximately 84 °C and 318 MPa after 30 min [117]. β-Glucoamylase from barley malt exhibited a maximum depolymerization rate at 215 MPa and 55 °C which yielded approximately 2/3 higher degradation of β-glucan after 20 min as compared to the maximum at 0.1 MPa and 45 °C [116]. Activity also increased 102 and 105% after treatment with SC-CO₂ and SC-CO₂ + 0.1 wt% water respectively at 35 °C and 20 MPa for 1 h compared to prior to treatment [182]. Like α-amylase, β-amylase, and β-glucanase discussed earlier, glucoamylase is widely used in the brewing industry and shows a clear activity and stability enhancement at elevated pressures. The
evidence of pressure enhancement of these four enzymes vital for beer and spirit production are examples of the potential improvement of industrial processes with HP.

**Lysozyme (EC. 3.2.1.17)**

Lysozyme is commonly found in nature and is primarily from egg albumin. Lysozyme hydrolyzes the β-1,4 glycosidic bond between the N-acetylmuraminic acid and the N-acetyl-D-glucosamine of peptidoglycan, which is the major component of gram-positive bacteria cell walls [186]. Lysozyme is relatively low-priced as compared to other antimicrobial agents such as nisin [187], is classified as generally recognized as safe (GRAS), and is effective as a food preservative by inhibition of pathogenic and spoilage bacteria [186, 188-191]. Lysozyme activity is relatively stable below 200 MPa while above 200 MPa some enzymatic activity is lost but antimicrobial activity was not reduced [186]. Luccie and others [192] also found that lysozyme treated at 100 MPa had increased antimicrobial activity resulting in approximately two logarithmic reductions in microbial death. The increase in antimicrobial activity was attributed to a direct effect of pressure on the integrity of cell walls or outer membranes of the microbes, a consequent increased penetration of enzymes through the damaged walls or membranes, and indirect effects on the processes of the antimicrobial properties of lysozyme [192]. While pressure increased the antimicrobial activity of lysozyme, it may be a synergistic or additive effect and not purely attributed to increased enzymatic activity. Although pressure effects on lysozyme activity above 200 MPa remain unclear, current evidence suggests that pressures below 200 MPa may aid in the action of this widely used, effective, and relatively inexpensive antimicrobial enzyme.
β-Glucosidase (EC. 3.2.1.21)

β-Glucosidase catalyzes the hydrolysis of aryl and alkyl β-D-glucosides and are involved in key metabolic events and growth related response in plants [193]. This enzyme is involved in the liberation of volatile aglycones from non-volatile glucosides [194] and is important to for subsequent flavor release [88]. In red raspberries, HPT at 400 MPa increased activity 2 % while in strawberries HPT of 400 MPa increased activity 76% [88]. β-glucosidase from Sulfolobus solfataricus was also activated by pressure between 0.1 and 250 MPa with maximal activity at 125 MPa and has a half-life of 91 h at 60 °C and 250 MPa. Conversely almond β-glucosidase was rapidly inactivated by pressure up to 150 MPa [195]. HP activation may be helpful in strawberry or raspberry processing where pressure is used to inactivate some unwanted enzymes while activating others that contribute to flavor development.

β-Galactosidase (EC. 3.2.1.23)

β-Galactosidases are widely used in the food industry to hydrolyze lactose into galactose and glucose [118]. Also, the transgalactosidase activity of β-galactosidases can be useful in synthesis of glyconjugates for food and pharmaceutical applications [196, 197]. β-galactosidases from Aspergillus oryzae, Kluyveromyces lactis, and Escherichia coli were investigated for their increased thermostability under HP. After 1 h incubation at 25 °C residual activity did not significantly decrease for β-galactosidase from A. oryzae below 450 MPa, below 300 MPa from E. coli, and below 200 MPa from K. lactis [118]. For the three enzymes tested, moderate pressures protected the enzyme against thermal inactivation. Residual activity of E. coli β-galactosidase increased from 60% at 0.1 MPa to 80% at 100 MPa and 55 °C [118]. Residual activity of K. lactis β-galactosidase increased 5-fold at 100 MPa versus at 0.1 MPa and 45 °C [118]. Most
interestingly, residual activity of *A. oryzae* β-galactosidase increased from complete inactivation at 55 °C and 0.1 MPa to near 100% residual activity at 250 MPa for a 1 h incubation [118]. β-galactosidase from *E. coli* had no decrease in activity below 250 MPa [198] and at 250 MPa the half life at 55 °C was 32 h versus 1.5 h at 0.1 MPa [199].

While application of HP to β-galactosidase in the food industry may be advantageous, as with many enzymes the application of HP for pharmaceutical synthesis of compounds such as glyconjugates may be more economically viable.

**Invertase (EC. 3.2.1.26)**

Invertase (β-D-fructofuranosidase) is widely used in the food industry for its hydrolytic activity on sucrose for the production of invert sugar (mixture of glucose, fructose, and unhydrolyzed sucrose) [200]. Pressure induced conformation changes were determined using Raman Spectroscopy [201] and fluorescence spectroscopy [202] where conformational changes induced by pressure and temperature were different. HP caused an increase in low-wave number scatter in solution and to a shift of the amide I bands lower while thermal denaturation showed no such modifications in spectra [201]. Likewise, thermal treatment of invertase caused the fluorescence of tyrosine and tryptophan to decrease slowly while HPT caused these aromatic residues to become more exposed causing increased fluorescence [202]. These differences were hypothesized to be due to HP and temperature exerting opposing effects on weak interactions (hydrogen bonds, ionic and hydrophobic interactions) leading to different denaturation mechanisms [201] and may explain the antagonistic effects of pressure and temperature for invertase pressure induced stabilization. *Saccharomyces cerevisiae* invertase half-life increased at 60 °C from 50 to 200 MPa [106]. Furthermore, native and immobilized invertase have $\Delta V^\neq = -7.3 \text{ cm}^3 \cdot \text{mol}^{-1}$ [203] and $\Delta V^\neq = -29 \text{ cm}^3 \cdot$
mol\(^{-1}\) [204] respectively. Conversely, invertase can be readily inactivated by pressure depending on the specific temperature-pressure combination [202, 205].

**Naringinase (EC. 3.2.1.40)**

Naringinase is used to reduce bitterness in grapefruit juice by hydrolyzing naringin (a flavonone glycoside and primary bitter component in grapefruit juice) to naringenin, which is tasteless. In a model solution, naringinase activity increased by 72% at 200 MPa and 54 °C versus only a 35% reduction at 0.1 MPa [122]. These results are in agreement with earlier findings where maximum naringinase activity was between 30 and 40 °C and approximately 160 MPa with a \(\Delta V^\neq = -9\) to \(-15.0\) cm\(^3\) mol\(^{-1}\) [119-121] depending on immobilization technique and other reaction conditions. The HP induced activation of naringinase makes it suitable for grapefruit juice processing as modified conditions can optimize naringinase activity while simultaneously inactivating undesirable pressure-labile microorganisms.

**Myrosinase (EC. 3.2.3.1)**

Glucosinolate hydrolysis products in broccoli catalyzed by myrosinase such as sulforaphane are thought to have beneficial health effects. Therefore, a heat and pressure treatment that results in stabilized myrosinase could be advantageous [124]. Inactivation rate constants decreased up to a maximum around 350 MPa at 35 °C. These results were later substantiated when thermal treatment of 60 °C reduced activity by 97% at 0.1 MPa versus 75% at 100 MPa [124]. The activation energy of myrosinase inactivation was highest at 200 MPa, the pressure at which the antagonistic effect was most pronounced [124]. This antagonistic effect was not observed with a second form of myrosinase for which inactivation rates continually increased with increased pressure [123]. The application of HP may effectively improve the inactivation of deleterious
enzymes and microbes while simultaneously increasing or preserving the activity of beneficial enzymes such as myrosinase. Although the relationship between myrosinase activity and pressure is not fully elucidated, it is clear that application of elevated pressure may be a useful tool in the enhancement of health benefits of processed broccoli.

**α-Chymotrypsin (EC. 3.4.21.1)**

α-Chymotrypsin (CT) is a proteolytic enzyme that facilitates the hydrolysis of peptide bonds and is used to aid protein digestion. It is a serine protease with the characteristic three amino acid active site known as the catalytic triad. Stability of α-chymotrypsin at HP was investigated in the 1940s [127]. While no pressure-induced activation was noted, induced stabilization was reported up to 760 MPa. Pressure effects on CT activity and stability were explored again in the 1980s by Taniguchi and Suzuki [128] and in more depth in the mid 1990s by Mozhaev and others [41, 125]. Taniguchi and Suzuki found that increasing pressure increased CT hydrolysis of p-nitrophenyl acetate from 10 to 65 °C with maximal activity at 45 °C and 200 MPa [128] and pressure activation was more pronounced as temperature increased. Similarly, Mozhaev and others found that an increase in pressure at 20 °C resulted in a 6.5-fold increase in activity at 470 MPa versus 0.1 MPa [125]. The acceleration effect became more pronounced at high temperatures as activity at 50 °C and 360 MPa was 30-fold higher than activity at 0.1 MPa and 20 °C [125]. High pressure also increased stability of CT. CT is readily inactivated (less than 5 min half-life) at 0.1 MPa; while at 180 MPa CT remains active for 30 min [125]. Stability and activity of CT was also improved by pressure in organic media when Mozhaev and others [126] obtained $\Delta V^\theta = -13 \pm 2$ cm$^3$ mol$^{-1}$ while maintaining activity up to 200 MPa. This was explained as being due to a
transitional conformation between 100 and 150 MPa that renders the enzyme more active. Raman spectroscopy was used to explore pressure-induced structural changes and found only slight changes up to 200 MPa that were attributed to the salt bridge between Asp-194 and Ile-16 [206]. While many enzymes native to muscle tissue are a target for inactivation by HP [207], the pressure-induced stabilization or activation of α-chymotrypsin may be useful in modifying the functional and organoleptic properties of a wide variety of processed meat products.

**Thermolysin (EC. 3.4.24.27)**

Thermolysin is used in the production of a precursor for a synthetic sweetener and may be suitable for the production of various peptides through the condensation of amino acids [208]. Thermolysin hydrolysis of β-lactoglobulin (BLG) rate increased by a factor of 22 from 0.1 to 200 MPa before irreversible conformational changes occurred at or above 300 MPa [129]. Similarly, BLG digestion by thermolysin increased from 19.4 to 25.0% at 200 MPa versus at 0.1 MPa [208]. In the same study thermolysin catalysis was accelerated by increasing pressure on nonspecific digestion of proteins from various origins. These proteins included, alcohol dehydrogenase (18.1% versus 45.3%), hemoglobin (13.0% versus 34.6%), bovine serum albumin (0.19% versus 0.23%), and myoglobin (12.0% versus 15.0%) at 0.1 MPa and 200 MPa respectively [208].

Increased reaction rate was attributed to pressure effects on enzymatic activation as well as protein substrate partial unfolding allowing greater catalytic activity on the protein substrate. This combined pressure effect is dually useful when conducting enzymatic catalyzed synthesis or hydrolysis reactions because pressure can act as a denaturation-inducing agent. This pressure effect on substrate denaturation was termed “reagent-less denaturant” by Kunugi [208].
A thermostable neutral thermolysin from *Bacillus thermoproteolyticus* was compared to a non-thermostable neutral thermolysin from *Bacillus subtilis* [209]. The application of 100 MPa resulted in over 13 times increase in reaction rate for the thermostable enzyme versus 3.8 times increase for the non-thermostable enzyme [209]. Also, thermolysin catalyzed hydrolysis of a dipeptide substrate (FA-Gly-Leu-NH₂) had a $\Delta V^\neq = -30.2 \text{ cm}^3 \text{ mol}^{-1}$ [210]. Thermolysin from *Bacillus thermoproteolyticus* was also shown to have $\Delta V^\neq = -52 \pm 4 \text{ cm}^3 \text{ mol}^{-1}$ and up to a 40-fold reaction acceleration at 100 MPa and 40 °C for hydrolysis of the low molecular mass substrate 3(2-furylacryloyl)-glycyl-L-leucine amide [211]. HP also increased residual activity by 101 and 102% after treatment with SC-CO₂ and SC-CO₂ + 0.1% water at 35 °C and 20 MPa for 1 h resulted compared to before treatment [182]. Similarly to lipase, thermolysin is used in high-value product (synthetic sweetener and peptide) biosynthesis; and therefore, use of HP technologies may be suitable due to the cost effectiveness associated with these products.

**Pepsin (EC. 3.4.23.1)**

Like thermolysin, pepsin is used as a catalyst in the production of a precursor for a synthetic sweetener and may be suitable for the production of various peptides through the condensation of amino acids [208]. The hydrolysis of BLG with pepsin was studied between 0.1 and 350 MPa. Increased pressure showed a significant increase in cleavage rates [129]. Pepsin was unable to hydrolyze BLG at 0.1 MPa versus total hydrolysis of all the BLG substrate in less than 40 min at 300 MPa [129]. The BLG peptic hydrolysis rate increased 270-fold between 0.1 and 300 MPa. Like lipase and thermolysin, pepsin can be used in high-value product biosynthesis. Therefore the use
of HP technologies may be suitable due to cost effectiveness associated with these products.

**Unidentified protease**

Proteases traditionally are applicable for the cleavage or digestion of proteins, which is particularly useful in the food or pharmaceutical industry. The properties of an unidentified and un-named protease from a hyperthermophilic barophilic *Methanococcus jannashii*, an extremely thermophilic deep-sea methanogen found at a deep-sea hydrothermal vent, have been explored. The activity and stability of the partially purified enzyme increased with pressure; raising the pressure increased the reaction rate 3.4-fold and the thermostability 2.7-fold at 50 MPa at 125 °C with a corresponding negative activation volume of $-106 \text{ cm}^3 \text{ mol}^{-1}$ [20] compared to 0.1 MPa. The barophilic behavior of this protease is somewhat expected in view of the unusual barophilicity exhibited by *M. jannashii* [212], as well as other enzymes from this organism such as hydrogenases discussed earlier [86, 87].

**Pyrophosphatase (3.6.1.1)**

Pyrophosphatases are acid anhydride hydrolases that act upon diphosphate bonds and are used in DNA polymerization reactions for *in vitro* RNA synthesis reactions and for pyrophosphate removal which is from biosynthetic reactions that utilize ATP [213, 214]. Inorganic pyrophosphatase from yeast was partially stabilized against thermal inactivation by the addition of trehalose and glycerol [215]. Partially purified pyrophosphatase from *Bacillus stearothermophilus* demonstrated increased activity at 90 °C from 10 to 100 MPa with optimal activity at 70 MPa [130]. No activity could be demonstrated at 100 °C at 0.1 MPa while at 105 °C and 103 to 190 MPa activity increased up to 320% compared to 0.1 MPa [130]. Activation was explained by
pressure counteracting the molecular volume increase which results from elevated temperatures. While this explanation is over-simplified, for the time of publication it was considered adequate. Like polymerase, the application of HP for conducting a DNA analysis may not be currently suitable with today's technology, although any method for stabilizing a widely used enzyme is worthy of further investigation.

**Lyases (EC 4)**

**Aspartase (4.3.1.1)**

Aspartase (*L-aspartate* ammonia-lyase) catalyzes the reversible deamination of the amino acid L-aspartic acid producing fumaric acid and ammonium ion [214, 216]. It was used to produce L-aspartic acid on a large scale [217, 218]. Aspartase of *Escherichia coli* was shown to be activated by pressure in the early 1960s as activity increased from 0.1 MPa to 100 MPa at 50 °C and from 0.1 MPa to 60 MPa at 53 °C and 56 °C [131]. It was suggested that the application of pressure may increase the incidence of substrate-active site collision as well as prevent enzyme unfolding. Like other early studies, this explanation was adequate for the time of publication but is understood to be an oversimplification.

**Remaining Challenges**

Although the effect of pressure on the stability and rates of reaction for many enzymes has been researched, there is still a lack of experimental data for the majority of known enzymes. Also, the effect of HP on enzyme structure is scarce because there are very few laboratories equipped with HP optical cells coupled to the instrumentation necessary for such studies. Currently, most methods require introducing enzyme to substrate then applying incubation conditions for a given time interval. After a given time, the enzyme reaction mixture is removed from the incubator and assayed, and
repeating this procedure at time intervals allows pseudo-apparent kinetic measurements. Although such efforts as precisely controlling temperature and enzyme to substrate ratios among other reaction parameters can effectively slow the reaction to give adequate apparent kinetic measurements, they must compensate for adiabatic heating and cooling and the pressurization and depressurization transient time. For these reasons, future studies are needed to develop in-situ kinetic measurements at HP with precise control of other reaction parameters such as temperature. Likewise, in-situ studies need to be conducted to fully elucidate quaternary, tertiary, and even secondary structural changes during pressure changes, at isobaric conditions, and at various combinations of temperature and pressure. These studies will allow greater understanding of enzymatic changes at elevated pressures which may allow for better utilization and optimization of reaction conditions.

Despite its advantages described here, the application of HP for enzymatic reactions may not always be practical due to the high cost of HP systems, lack of appreciable effects, or advancements in alternative enzyme enhancement technologies. However HP has great potential and is only beginning to be explored in the field of enhancing enzyme systems.

**Specific Objectives**

The central hypothesis of this research is that high pressure can be used to increase lipase activity and stability. The overall objective of this research is to document the effects of high hydrostatic pressure on free and immobilized lipase catalyzed isoamyl acetate synthesis in selected solvents. The first specific objective is to determine the stabilization and activation effects of HHP on immobilized lipase in hexane. The working hypothesis is that high pressure will increase immobilized lipase
activity and stability in hexane. This objective will be achieved by determination of several parameters; including $\Delta V^\ddagger$, $E_a$, $V_{max}$, and $K_M$ while also observing the effects of HHP on the immobilization support. The second specific objective is to determine the effect of HHP on immobilized and free lipase catalyzed synthesis of isoamyl acetate in an ionic liquid-alcoholic biphasic mixture. The working hypothesis of this specific objective is that the combination of using IL-alcohol biphasic mixtures and HHP will provide a new method of increasing the reaction rate of free and immobilized lipase. To achieve this second specific objective it is necessary to determine the $\Delta V^\ddagger$, $E_a$. The last specific objective is to continuously monitor *in situ* conformational studies of free lipase as it is subjected to thermal and HHP treatment. The working hypothesis of the third specific objective is that conformational changes occur within the lipase at HHP and are different depending on the temperature-pressure combination. This specific objective will be achieved by recording fluorescence intensity changes induced by changes in the tertiary structure of the lipase (mainly tryptophan).
Figure 1-1. Theoretical generic elliptical pressure versus temperature diagram with native and denatured protein regions inside and outside of the ellipse respectively, separated by a zone of reversible denaturation.
CHAPTER 2
HIGH HYDROSTATIC PRESSURE INCREASED STABILITY AND ACTIVITY OF IMMOBILIZED LIPASE IN HEXANE

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 [219] and ability to be considered “natural” [220]. 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 [38, 42, 45, 52, 67, 161, 221, 222]. Lipases are widely used in industrial applications because of their high enantioselectivity, wide range of substrates, thermal stability, and stability in organic solvents [223]. Isoamyl acetate esterification catalyzed by lipases from various sources has been studied in a wide variety of solvents [38, 52, 161], kinetic studies have proposed a Ping-Pong Bi-Bi mechanism [221], and operating conditions such as acyl donor, type of lipase, and temperature have been optimized [38]. 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 [8-14], its effects on enzyme stabilization and activation have been documented relatively little [15-19]. HHP has been shown to stabilize or activate chymotrypsin [125], naringinase [120, 121], polyphenol oxidase [13], pectin methylesterase [13, 97, 99], and more extensively lipases in dense gases and solvent free systems [50, 104, 109, 110, 159,
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 [17-19, 28]. This antagonistic relationship results in an elliptical pressure vs. temperature diagram with native and denatured protein regions inside and outside of the ellipse, respectively [17]. 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 [17-19, 28] 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 [38]. 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 [22-24]. However, the boiling point of most organic solvents is well below 100 °C. For example hexane boils at 67.8 °C (at 0.1 MPa) which restricts studies in hexane to be explored below 65 °C. As
pressure is raised the boiling point of organic solvents also increases allowing studies to examine activity above conventional temperatures at low pressure [25]. 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 [26] 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 2008 [27]. As demand for HPP equipment grows, innovation is expected to continue to reduce capital and operating costs [26]. 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.

**Materials and Methods**

**Materials**

Lipase (Novozyme 435® E.C. 3.1.1.3) from *Candida antarctica* lipase B (CALB) expressed in *Aspergillus oryzae* immobilized on a macro porous 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 MP5), and a pump controller (MP5 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); one cooling (5 or 10 °C) and another heating (25 to 80 °C ± 0.1 °C) from Fisher Scientific and controlled by an array of pinch valves. Computer programs written in LabVIEW and a data acquisition board (DAQ Card 6062E) with a signal conditioner (model SC-2345) from National Instruments (Austin, TX USA) were used to collect temperature and pressure data and to control the heating/cooling valve array. The HHP system is depicted in Fig. 2-1 and shown in Appendix 1. Stirring inside the reaction vial was initiated by a magnetic stir-bar inside the reaction vessel and controlled by external spinning neodymium magnets on an AC motor type NS1-12 (Bodine Electric Company, Chicago, IL USA). Reaction progress was monitored using GC-FID 5890 (HP, Palo Alto, CA USA) with a ZB-5 column (30 m length × .53 mm ID × 1.5 μm thickness) at a gradient temperature from 50 °C to 90 °C at 5 °C min⁻¹. Injector temperature was held at 200 °C and FID detector at 250 °C. The scanning electron microscope (SEM) was a Hitachi model S-530 (Tokyo Japan). Samples were coated using a Ladd (Williston, VT USA) sputter coater.

Methods

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 Leur-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 [38, 221] and shown in equation 2-1.

\[
\begin{align*}
\text{Isoamyl Alcohol} & \quad + \quad \text{Acetic Acid} \\
\rightarrow & \quad \text{Lipase} \\
\text{Isoamyl Acetate} & \quad + \quad \text{H}_2\text{O}
\end{align*}
\]

(2-1)

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 though the luer-lok™. 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 [38, 42, 221], 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 ºC (hexane boiling point at ambient pressure) pressure had 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 ºC for 4 hr (data not shown) which was similar to previous studies [224] whereby 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).

Comparison of application of HHP and heat methods

Three sequences of application of pressure and heat were utilized to compare changes in enzyme activity derived from non-inactivating conformational enzyme changes. Such changes were assessed thought the determination of rate constants and
activation energies. Application sequences were pressurization first, simultaneous heating and pressurization, and heating first as shown in Fig. 2-2. 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 to 400 MPa) and temperature (36, 42, 48.9, 56.2, 63.5, 71.8, 80 ± 0.1 °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 °C ± 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 vs. time. Reaction rate was expressed as concentration (mmol L⁻¹) 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 E_a. Statistical comparisons of treatment methods were conducted using SAS statistical software (Cary, NC USA) utilizing GLM regression analysis.
Activation volume

Pressure effects on initial rate were examined at 0.1 to 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 ($R^2 > 0.95$) of the time course production of isoamyl acetate by fitting product concentration vs. time. Reaction rate was expressed as concentration (mmol L$^{-1}$) 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 ($\Delta V^\#$) determined from Eyring’s equation (shown in Chapter 1, equation 1-1).

where $k$ is the equilibrium constant, $p$ the pressure, $T$ the absolute temperature, $R$ the gas constant and $\Delta V^\#$ the activation volume [225].

Electron micrographs of immobilization beads

Scanning electron microscopy (SEM) was utilized to examine the solvent, temperature, and pressure effects on the immobilization support (macro porous 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.
Results and Discussion

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. 2-3. 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%. Pairwise 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 [226]. 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 [169], derivative spectroscopy [227], x-ray scattering [228], and quantitative Raman spectroscopy [229]. To conduct these studies
with CALB, the enzyme must be solubilized in hexane using a synthetic detergent according to Tsuzuki et al.[230], 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* [106]. At denaturing temperatures (50, 55, and 60 °C) application of pressure protected the lipase at a range of 50 to 350 MPa by increasing residual activity from about 82 to 95% at 50 °C, about 32 to 70% at 55 °C, and from 0 to about 35% at 60 °C [106]. 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 [106]. This phenomenon is also similar to that of pectin methyl esterase (PME) which showed an increase in optimal temperature from 45 to 55 ºC as the pressure increased from 0.1 MPa to 300-500 MPa) [99].

These stability studies indicate that pressure significantly stabilizes the immobilized lipase up to 400 MPa. Recently, the activity of lipases from porcine pancreas, *Candida antarctica*, *Candida cylindracea* (immobilized), *Penicillium roqueforti*, *Aspergillus niger*, *Rhizopus arhizus*, *Mucor miehei*, and *Pseudomonas cepacia* was studied after hydrolysis under SC-CO\(_2\) at 40 °C and 15 MPa [171]. All lipases tested had increased relative activity with the greatest increase from *Rhizopus arhizus* lipase which was increased by more than 50 times while *Candida antarctica* lipase activity increased
from 0.32 to 5.97 U g\(^{-1}\) after treatment in SC-CO\(_2\) and up to 21.00 U g\(^{-1}\) during exposure to SC-CO\(_2\). However, these results contrast to previous studies of lipase in supercritical carbon dioxide (SC-CO\(_2\)) and compressed n-butane which showed increased activity as pressure increased up to 35 MPa [50, 104, 109] 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 Mozhaev et al. [125], 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 [30]. 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 [91]. 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 [231]. Hydrogen bridges are generally known to be stabilized by pressure and destabilized by heat [231, 232]. 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 \textit{R. miehei} in aqueous solution. According to Noel et al [169] 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 et al. [169] 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 [169].

**Comparison of Pressurization and Heating Methods on Activation Energy**

Initial rate of isoamyl acetate production using each method at 400 MPa and 36 to 80 °C was compared to assays at low pressure. Apparent initial rate results are shown in Table 2-1 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 to 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 to 63.5 °C. $E_a$ at low pressure and was $42.7 \pm 3.60 \text{ kJ mol}^{-1}$. The pressure-first, simultaneous, and heat-first methods had $E_a$ of $42.36 \pm 6.9$, $47.8 \pm 8.16$, and $35.7 \pm 3.5 \text{ kJ mol}^{-1}$ 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. [91] 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 2-1. 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 acetic anhydride (a more effective acyl donor) resulted in lower $E_a$ (11.3 kJ mol$^{-1}$) [38] for synthesis of isoamyl acetate. $E_a$ was slightly higher to that reported in a solvent free system (28.7 kJ mol$^{-1}$) [47] and in biphasic-ionic liquid (27.3 kJ mol$^{-1}$) [52]. These results are similar to other ester synthesis reactions catalyzed by CALB; including butyl butyrate (32 kJ mol$^{-1}$) in SC-CO$_2$ [157] and tetrahydrofurfuryl butyrate (47 kJ mol$^{-1}$) [233], oleyl oleate (37 kJ mol$^{-1}$) [234], butyl laurate (45 kJ kJ mol$^{-1}$) [234], butyl oleate (37 kJ kJ mol$^{-1}$) [234], and oleyl butanoate in hexane (39 kJ mol$^{-1}$) [234].

Several enzymes have shown enhanced activity while or after exposure to HHP; including polyphenoloxidase [88, 96], pectin methylesterase [97, 99], α-chymotripsin [125], dehydrogenase [83, 84], protease [20], α-amylase [114], peroxidase [88], thermolysis [129], and pepsin [129] 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 [56]. However, at least fifteen dimeric and tetrameric enzymes that are activated by pressure have been reported [32]. Noel et al. [169] 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.

**Activation Volume Determination**

Effect of pressure on the initial rate \( V_{\text{max}} \) of lipase catalyzed isoamyl acetate formation at 40 and 80 °C is shown in Fig. 2-4. Activity was higher at 80 °C than at 40 °C at all pressures. At 80 °C \( V_{\text{max}} \) increased with pressure in the range of 0.1 to 300 MPa followed by a decrease in the range of 350 to 600 MPa. At 40 °C \( V_{\text{max}} \) increased with pressure in the range of 0.1 to 100 MPa followed by a decrease in the range of 200 to 600 MPa. Activities at 100 to 400 MPa at 40 °C and at 100 to 450 MPa were higher than at low pressure. Maximum rate of reaction was \( 2.09 \times 10^{-3} \) at 300 MPa and 80 °C. This pressure was below that of maximum stability (400 MPa) discussed previously. 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. This provides insight into the effects of the presence or absence of substrate on lipase stabilization.

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 on the reaction rate was described using the Eyring equation to determine $\Delta V^\neq$ as shown in Fig. 5. $\Delta V^\neq$ slightly increased with temperature from 14.3 ± 1.7 to 15.2 ± 2.2 cm$^3$ mol$^{-1}$ at 40 or 80 °C, between 300 and 500 MPa. These results were not significantly different. Interestingly, apparent $\Delta V^\neq$ was negative from 0.1-200 MPa at 80 °C and from 0.1-100 MPa at 40 °C. At pressure below 400 MPa inactivation occurred at 80 °C as previously shown in Fig. 2-3. Therefore, at 80 °C below 400 MPa the apparent negative $\Delta V^\neq$ 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 whereby the lipase is considered stable [38]. Therefore, both enzyme stabilization and pressure activation might be occurring simultaneously. Recently carrot pectin methyl-esterase (PME) showed very similar effects while at range of temperature (30–60 °C) and pressures (0.1–600 MPa) [101]. The PME showed a negative $\Delta V^\neq$ below 400 MPa at temperature greater than 60 °C and a positive $\Delta V^\neq$ above 400 MPa at all temperatures tested [101].

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-CO$_2$ on the isoamyl acetate synthesis from isoamyl alcohol using CALB was reported by Romero et al. [161]. 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, *Pseudomonas cepacia* lipase (PCL) catalyzed transesterification of 1-phenylethanol with vinyl acetate in SC-CO$_2$ showed an increase in catalytic efficiency up to 15 MPa. However, $\Delta V^\circ$ did vary with pressure range used, reaching a maximum negative value of $-1340$ cm$^3$ mol$^{-1}$ at 7.4 MPa [55]. High negative $\Delta V^\circ$ would be expected in SC-CO$_2$ 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 $\alpha/\beta$ hydrolase fold family with a catalytic triad consisting of Ser, His, and Asp/Glu [172]. Early X-ray diffraction data suggests that a lipase’s helical part of the $\alpha$-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 [173]. 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 [68] as compared to *Candida antarctica* lipase A (CALA) which does possess the characteristic “lid” structure and shows interfacial activation [174]. Furthermore, flexibility of CALB is reduced in organic solvents, and is limited to: (1) a short $\alpha$-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 [68]. 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 [16, 18, 19, 28-31, 235]. It is understood that pressure effects on catalysis reactions are related to Le Chatelier’s principle: elevated pressures favor changes that reduce a systems 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 [28]. 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 cm$^3$ mol$^{-1}$ to 60 cm$^3$ mol$^{-1}$ with most being less than 30 cm$^3$ mol$^{-1}$ [28] 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%) [66]. 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 micro aqueous layer of the enzyme [67]. 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 [68]. Therefore, pressure may increase flexibility of CALB.

Lipase activity in organic solvents was correlated with the solvent polarity [40]. 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 [64, 73]. 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.

**Electron Microscope Examination of Immobilization Beads**

In an effort to isolate HHP effects on lipase versus the effects on the immobilization support, scanning electron microscope (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 (macro porous 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 occurred in either the overall shape or in the surface texture of the immobilization bead as compared to the control and to previous studies [226]. Surface differences do appear in Fig. 6A and 6B due to orientation of the bead during imaging. Also, differences can be seen when comparing Fig. 6A, B and Fig. 6C, 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 towards 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 [228, 229] which allow continuous monitoring.

**Conclusions**

Previous studies [38, 42, 221] 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

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Figure 2-2. Temperature (circle) and pressure (triangle) profiles for (A) “Pressure-First”, (B) “Simultaneous”, and (C) “Heat-First” method for a 10 minute incubation at 80 °C and 400 MPa.
Figure 2-1. Schematic representation of the high hydrostatic pressure system.

Figure 2-3. 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.
Figure 2-4. Effect of pressure on $V_{\text{max}}$ at (●) 40 °C and (■) 80 °C for lipase-catalyzed production of isoamyl acetate (Novozyme 435 in n-hexane) using the "pressure-first method". Error bars represent standard error of the slope of apparent the initial rate calculated from linear regression using a 95% confidence intervals.

Figure 2-5. Determination of activation volume using the Eyring equation at (●) 40 and (■) 80 °C for lipase catalyzed production of isoamyl acetate using the "pressure-first method".
Figure 2-6. 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.
Table 2-1. 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>Pressure First (400 MPa)</th>
<th>Heat First (400 MPa)</th>
<th>Simultaneous (400 MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Rate (mmol L⁻¹ sec⁻¹)</td>
<td>Pressure (MPa)</td>
<td>Initial Rate (mmol L⁻¹ sec⁻¹)</td>
<td>% Change</td>
</tr>
<tr>
<td>36.0</td>
<td>5.31 x 10⁻³</td>
<td>0.1</td>
<td>4.49 x 10⁻³</td>
<td>-15.4</td>
</tr>
<tr>
<td>42.0</td>
<td>7.45 x 10⁻³</td>
<td>1</td>
<td>4.50 x 10⁻³</td>
<td>-39.6</td>
</tr>
<tr>
<td>48.9</td>
<td>1.00 x 10⁻²</td>
<td>2</td>
<td>9.32 x 10⁻³</td>
<td>-6.8</td>
</tr>
<tr>
<td>56.2</td>
<td>1.69 x 10⁻²</td>
<td>6</td>
<td>1.02 x 10⁻²</td>
<td>-40.4</td>
</tr>
<tr>
<td>63.5</td>
<td>1.94 x 10⁻²</td>
<td>7</td>
<td>1.62 x 10⁻²</td>
<td>-16.3</td>
</tr>
<tr>
<td>71.8</td>
<td>1.60 x 10⁻²</td>
<td>8</td>
<td>1.91 x 10⁻²</td>
<td><strong>19.8</strong></td>
</tr>
<tr>
<td>80.0</td>
<td>1.57 x 10⁻²</td>
<td>10</td>
<td>2.49 x 10⁻²</td>
<td><strong>43.2</strong></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.
CHAPTER 3  
ACTIVATION VOLUME AND APPARENT MICHAELIS MENTEN PARAMETERS OF LIPASE AT HIGH HYDROSTATIC PRESSURE IN HEXANE WITH EXCESS ALCOHOL

Introduction

Kinetic studies of lipase-catalyzed reactions in hexane have proposed a Ping-Pong Bi-Bi mechanism shown in Equation 3-1 [221], and operating conditions such as acyl donor, type of lipase, and temperature have been optimized [38]. Also, evidence of HP enzyme enhancement and possible mechanisms of stabilization and activation have been reviewed and discussed in chapter 1. Although chapter 2 has documented the activation energies ($E_a$) and activation volume ($\Delta V^\ddagger$) of inactivation, no work has focused on investigating the $\Delta V^\ddagger$ of activation or other kinetic parameters of lipase catalyzed synthesis of isoamyl acetate in an organic solvent at HHP. Kinetic parameters are necessary for further optimization of temperature and pressure conditions while utilizing lipase catalyzed synthesis at elevated pressure. Furthermore, by characterizing the $\Delta V^\ddagger$ of activation the mechanism of pressure induced activation can be explored further. The objective of this research was to determine the effects of pressure on increasing the overall reaction rate and on enzyme-substrate (acetic acid) binding in excess alcohol. To achieve this objective the apparent Michaelis Menten parameters and the $\Delta V^\ddagger$ of activation for the synthesis of isoamyl acetate in hexane catalyzed by immobilized lipase at HHP was determined.

Materials and Methods

Materials

Lipase (Novozyme 435® E.C. 3.1.1.3) from Candida antarctica lipase B (CALB) expressed in Aspergillus oryzae immobilized on a macro porous 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 MP5), and a pump controller (MP5 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); one cooling (5 or 10 °C) and another heating (25 to 80 °C ± 0.1 °C) from Fisher Scientific and controlled by an array of pinch valves. Computer programs written in LabVIEW and a data acquisition board (DAQ Card 6062E) from National Instruments (Austin, TX USA) were used to collect temperature and pressure data and to control the heating/cooling valve array. A depiction of the HHP system has been previously described in chapter 2 and is pictured in Appendix 1. Stirring inside the reaction vial was initiated by a magnetic stir-bar inside the reaction vessel and controlled by external spinning neodymium magnets on an AC motor type NS1-12 (Bodine Electric Company, Chicago, IL USA). Reaction progress was monitored using GC-FID 5890 (HP, Palo Alto, CA USA) with a ZB-5 column (30 m length × .53 mm ID × 1.5 μm thickness) at a gradient temperature from 50 °C to 90 °C at 5 °C min⁻¹. Injector temperature was held at 200 °C and FID detector at 250 °C.
Methods

Determination of lipase catalyzed isoamyl acetate production rate

Enzyme was weighed (2.5 mg or ~128 Propyl Laureate Units) into the reaction vial with a miniature stir bar. The reaction mixture (solvent and substrates, described in sections 2.2.2 and 2.2.3) was added to the reaction vial to initiate the reaction. The reaction vial plunger was moved into position to eliminate air bubbles then sealed with a Leur-lock™ plug. The reaction vial was then placed in the high pressure reaction chamber being held at 5 °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 the set-point. Upon completion of incubation, the temperature was returned to 5 °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 [38, 168, 221] and shown as

Pressure and temperature treatments were applied using the "pressure-first" method shown in Fig 3-1. and using the high pressure system previously described [168].

Determination of activation volume

Pressure effects on initial rate were examined between low pressure (between 1 and 10 MPa) and 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 \( R^2 > 0.95 \) of the time course
production of isoamyl acetate by fitting product concentration vs. time. The effects of pressure on enzyme activity were described by an overall activation volume (ΔV^≠) determined from Eyring’s equation (shown in Chapter 1, equation 1-1).

**Determination of apparent Michaelis-Menten parameters**

Apparent \( K_M \) and \( V_{\text{max}} \) were determined at low pressures (between 1 and 10 MPa) and at 200 MPa at 40 °C or 80 °C. Initial rate was determined by mixing 10 ± 0.1 mg immobilized lipase and isoamyl alcohol and acetic acid in hexane in the reaction vial and immediately inserting it into the reactor. Isoamyl alcohol concentration remained constant (5.0 X 10^{-1} M, in excess) while the acetic acid concentration varied from 5.0 X 10^{-3} to 2.5 X 10^{-1} M. Rate was determined from the slope using linear regression (\( R^2 > 0.95 \)) of the time course production of isoamyl acetate by fitting product concentration vs. time. Apparent \( K_M \) and \( V_{\text{max}} \) were determining using Lineweaver-Burk approximation.

Although conventional synthesis of isoamyl acetate is conducted at acid-alcohol ratios of 1:1 [221], in this study excess of non-inhibitory isoamyl alcohol is utilized to determine the apparent \( K_M \) and \( V_{\text{max}} \) by reducing the Ping-Pong Bi-Bi mechanism reaction shown in Figure 3-2 to an apparent one substrate, one product Michaelis Menten kinetics.

**Results and Discussion**

**Activation Volume**

The effect of pressure on the initial rate (\( V_{\text{max}} \)) of lipase catalyzed isoamyl acetate formation at 40 and 80 °C is shown in Fig. 3-3. Activity was higher at 80 °C than at 40 °C at all pressures. Activity was also higher at elevated pressures than at low pressure up to 400 MPa at 80 °C and up to 350 MPa at 40 °C. At 80 °C initial rate increased from 10 to 250 MPa, remained relatively constant between 250 and 350 MPa, then
decreased from 350 to 600 MPa. At 40 °C $V_{\text{max}}$ increased from 1 to 100 MPa, remained relatively constant between 100 to 200 MPa, and then decreased from 200 to 600 MPa.

The relationship between pressure and the logarithm of $V_{\text{max}}$ was determined in the linear ranges between 1 to 100 MPa at 40 °C and between 10 to 150 MPa at 80 °C as shown in Fig. 3-4. The $\Delta V^\dagger$ values are summarized in table 3-1. For both temperatures increasing pressure initially increased reaction rates to a point where higher pressures decreased reaction rates as indicated by negative and positive $\Delta V^\dagger$ values respectively. Temperature did affect $\Delta V^\dagger$ at low pressure but did not at high pressure (as shown in Chapter 2); indicating that pressure induced activation is affected by temperature while inactivation is not. This may be explained by pressure weakening or strengthening intra or intermolecular bonds or modifying vital structural motifs. These modifications can lead to either greater exposure (i.e. activation at low pressure), or concealment (i.e. inhibition at higher pressure) of the active site. For example, as discussed in chapter 1, hydrogen bonds are weakened by high temperature and strengthened by high pressure. Therefore, at lower temperature, this strengthening of hydrogen bonds as pressure increases (from 0.1 to 100 MPa) may have a pronounced effect on the active site. This effect would then be diminished by the disruption effects of elevated temperature (at 80 °C) and result in a less negative $\Delta V^\dagger$. Likewise, the effect of increasing pressure above 300 MPa may have overriding effects on structurally vital bonds that result in the same positive $\Delta V^\dagger$ regardless of temperature.

The negative $\Delta V^\dagger$ found in this study is similar to another lipase from *Pseudomonas cepacia* (PCL). PCL catalyzed transesterification of 1-phenylethanol with
vinyl acetate in SC-CO$_2$ showed an increase in catalytic efficiency up to 15 MPa. Also, \( \Delta V^\# \) did vary with pressure range used, reaching a maximum negative value of -1340 cm$^3$ mol$^{-1}$ at 7.4 MPa [55]. High negative \( \Delta V^\# \) would be expected in SC-CO$_2$ 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.

Because the activation energy (\( E_a \)) is constant regardless of the pressure (as shown in Chapter 2), to properly model the effects of pressure and temperature on reaction rate simultaneously, a function of \( \Delta V^\# \) \textit{versus} temperature is required. Such functions have been proposed and generated for thermal and pressure inactivation of PPO by Weemaes and others [91] and for actinidin by Katsaros and others [236]. A polynomial function describing the effects of pressure on rate at different temperatures was substituted into the Arrhenius equation. According to Weemaes, the proposed model accurately described the empirical data within the range tested. However, in contrast to this study, both Weemaes and Katsaros found that \( E_a \) of inactivation was affected by changes in pressure. These differences in pressure-temperature effects may be attributed to different enzymes and differences in the temperature-pressure combinations. Therefore, as a general rule it must be considered that over a broad enough temperature or pressure range, changing temperatures will always affect \( \Delta V^\# \) and changing pressures will always affect \( E_a \).

\textbf{Michaelis-Menten Parameters}

Effect of pressure and temperature on the Michaelis-Menten parameters \( K_M \) and \( V_{\text{max}} \) at 40 or 80 °C and low pressure or 200 MPa is shown in Table 3-2 and in Fig 3-5.
Pressure significantly increased $V_{\text{max}}$ at both temperatures by approximately 10-fold. Pressure induced increases in $V_{\text{max}}$ is in agreement with previous results (chapter 2) that demonstrated the effect of pressure induced rate increases on lipase catalyzed isoamyl acetate synthesis in hexane. $V_{\text{max}}$ was greater at 40 °C and 200 MPa than at 80 °C and 0.1 MPa. The lipase may be undergoing thermal induced inactivation at 80 °C and 0.1 MPa since according to the results in chapter 2, at pressure below 400 MPa, lipase inactivation occurs at 80 °C.

Increasing pressure increased $K_M$ at 40 °C by 14-fold which indicates a decrease in affinity between the CALB and acetic acid. Although this decrease in affinity can slow the overall reaction velocity, these effects are lessened or null when pressure simultaneously increases the product release ($V_{\text{max}}$, which is typically the slow/limiting step). At 80 °C $K_M$ was not affected by pressure which indicates that the formation of the enzyme-acid complex is being hindered at 40 °C but not at 80 °C. The conformational changes that account for decreased enzyme-substrate affinity appear to be nullified by increased temperatures. The dissociation of the enzyme-product complex ($k_2$ in Equation 1-3, Chapter 1) is typically the limiting step in catalysis and is often referred to as the apparent rate of catalysis. However, at 40 °C pressure affected the substrate binding ($k_1/k_{-1}$ in Equation 1-3, or $K_M$ in Equation 1-4 Chapter 1), thus altering the limiting step. The pressure dependence of the Michaelis Menten constant can be determined and used to obtain the so-called reaction volume ($\Delta V_{K_M}$) shown in equation 3-1 [28].

$$\Delta V_{K_M} = RT \left( \frac{\partial \ln K_M}{\partial P} \right)_T$$

(3-1)
Pressure effects on substrate binding are thought to be the primary cause of pressure sensitivity for several enzymatic reactions. According to Michels and Clark [28], the $\Delta V_{KM}$ can vary dramatically, and even change sign, depending on the substrate. In other words, increasing pressure can increase or decrease the affinity of the substrate to the enzyme. However, because an increased $K_M$ was only observed at 200 MPa and 40 °C, the inhibitory effect of pressure on substrate binding may be overcome with elevated temperature. Furthermore, since $V_{max}$ increased with pressure regardless of temperature, any changes in substrate binding ($K_M$) are overshadowed by increases in rate of the limiting step ($k_2$).

Several assumptions have been made to determine the $K_M$ and $V_{max}$ of lipase catalyzed isoamyl acetate formation while in excess alcohol. These assumptions reduce the Ping-Pong Bi Bi mechanism shown in Fig 3-1 to a simple one substrate one product Michaelis Menten equation. It is assumed that alcohol-enzyme binding ($K_{M2}$) is faster than acid-enzyme binding ($K_{M1}$) meaning that $K_{M2}$ is not a limiting step. Therefore $K_{M1}$ is representative of the overall $K_M$. It is also assumed that $K_{M2}$ is not affected by the concentration of acid. These assumptions reduce $K_{M1}$ and $K_{M2}$ to just $K_M$. Furthermore, it is assumed that the release of the ester ($k_{cat2}$) is the overall slow/limiting rate and that the release of water ($k_{cat1}$) is not affected by the concentration of acid. Therefore $k_{cat2}$ is representative of the overall $k_{cat}$ ($k_2$ from equation 1-3). Lastly, it is assumed that isoamyl alcohol (as a substrate) provides no inhibitory action regardless of concentration. These assumptions are not made arbitrarily. Evidence has been provided that isoamyl alcohol has no inhibitory effects on lipase catalyzed synthesis of isoamyl acetate, acetic acid is only inhibitory above 100 mM (this study used 60 mM), and that
lipase has a greater affinity for acetic acid than isoamyl alcohol [221]. These findings confirmed the Ping-Pong Bi Bi mechanism shown in Fig 3-1, but did not address the assumption that the release of the final product ester (k_{cat2}) is the overall slow/limiting rate. However, in a mono-phasic anhydrous solvent system such as hexane, it is typically understood that water release occurs rapidly due to equilibrium effects and its release does significantly affect enzyme conformation due to dilution effects.

Pressure effects on lipase catalyzed reactions have been previously discussed (in chapter 2) and have elaborated on the complexities of explaining pressure induced activation. Pressure induced changes in activity or stability are most likely attributed to one or more of the following; 1) shifts in tertiary structure of the lipase (thus affecting exposure to active site, stability characteristics, etc.), 2) changes in solvent polarity (affecting lipase tertiary structure and/or flexibility), 3) changes in substrate or solvent molar volume (thus affecting viscosity and substrate concentration, 4) changes in the limiting step, and 5) changes in chemical equilibrium rates of the reaction. Although it is not completely clear, changes in K_M provide evidence that pressure induced activation may be related to changes in the tertiary structure of the lipase and/or the limiting steps of the reaction.

Conclusions

This study provides evidence that increasing pressure can inhibit substrate (acetic acid) to enzyme binding (K_M), while increasing temperature overcomes this effect by increasing V_{max}. It also provides evidence that pressure increases the reaction rate of lipase differently depending on the temperature within a define pressure range. These findings contribute to future optimization of isoamyl acetate production studies by
providing the framework for proper pressure-temperature combinations and substrate concentrations.

Acknowledgments

This project was supported by a Research Innovation Grant from the University of Florida Institute of Food and Agricultural Sciences.

Figure 3-1. Temperature (○) and pressure (▲) profiles using the “pressure-first” method at 80 °C and 400 MPa.

Figure 3-2. Kinetic mechanism (Ping Pong Bi Bi) of lipase-catalyzed synthesis of isoamyl acetate from acetic acid and isoamyl alcohol using Cleland’s notation where E represents free enzyme and E* is the activated enzyme state.
Figure 3-3. Effect of pressure on $V_{\text{max}}$ at (●) 40 °C and (■) 80 °C for lipase-catalyzed production of isoamyl acetate (Novozyme 435 in n-hexane). Error bars represent standard error of the slope of apparent initial rate calculated from linear regression using a 95% confidence interval.

Figure 3-4. Linearized effect of pressure on $V_0$ according to Eyring equation at (●) 40 and (■) 80 °C for lipase catalyzed production of isoamyl acetate.
Figure 3-5. Lineweaver-Burk plot of isoamyl acetate production at (♦) 0.1 MPa and 40 °C, (▪) 200 MPa and 40 °C, (▲) 0.1 and 80 °C, and at (●) 200 MPa and 80 °C. Rate is expressed as mmol isoamyl acetate L⁻¹ s⁻¹ while substrate concentration is expressed as mmol L⁻¹ acetic acid.

Table 3-1. Activation volume using the Eyring equation at 40 and 80 °C for lipase catalyzed production of isoamyl acetate. ± Values represent the standard error of the linear regression used in determination of activation volume.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Pressure (MPa)</th>
<th>ΔV‡ (cm³ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>1-100</td>
<td>-21.6 ± 2.9</td>
</tr>
<tr>
<td>80</td>
<td>10-125</td>
<td>-12.9 ± 1.7</td>
</tr>
</tbody>
</table>

Table 3-2. Michaelis-Menten kinetic parameters $K_M$ (mmol L⁻¹) and $V_{max}$ (mmol L⁻¹ s⁻¹) of lipase catalyzed isoamyl acetate formation in hexane at 40 or 80 °C and 0.1 or 200 MPa. The ± values represent standard error from linear regression.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Pressure (MPa)</th>
<th>$K_M$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.1</td>
<td>2.4 ± 0.004</td>
<td>7.7E-3 ± 2.0E-3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>38.0 ± 0.684</td>
<td>7.3E-2 ± 1.8E-2</td>
</tr>
<tr>
<td>80</td>
<td>0.1</td>
<td>4.7 ± 0.094</td>
<td>4.3E-2 ± 2.0E-2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6.2 ± 1.303</td>
<td>4.2E-1 ± 2.1E-1</td>
</tr>
</tbody>
</table>
CHAPTER 4
ENHANCEMENT OF NATURAL FLAVOR SYNTHESIS USING HIGH PRESSURE AND AN IONIC LIQUID-ALCHOL BIPHASIC MEDIA

Introduction

Room temperature ionic liquids (ILs) are liquids that are composed completely of ions and are liquid at or near room temperature [237]. They have garnered much recent attention due to their unique properties as a reaction medium. They can have near-zero vapor pressure (the pressure of a vapor in thermodynamic equilibrium with its condensed phase), high thermal stability, and widely tunable properties (by changing the cation/anion combination) such as polarity, hydrophobicity, and solvent miscibility [237]. ILs are attractive reaction media because their use can enhance enzyme stability [53, 54, 238, 239], selectivity [240], and reaction rates [238, 241]. Also, as compared to mono-phasic systems, ILs utilized in a bi-phasic system can improve downstream processing and separation operations when using free enzymes, thus improving production efficiency [160, 237, 242]. Production efficiency is improved by helping facilitate the physical separation of the reactant and product layer while leaving the enzyme-IL layer unadulterated. Furthermore, enzymes in ILs have been termed as “carrier-free” immobilized [243]. Like traditional carrier-bound immobilization, carrier-free immobilization is also a method of modifying an enzyme to enable enzyme, solvent, substrate, or product separations, extractions, or recycling to occur without loss of enzyme activity. The main difference is that carrier-bound immobilization requires the enzyme to be bound to a non-catalytic support while carrier-free does not. Types of carrier-free immobilization include cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs), and cross-linked dissolved enzymes (CLEs). Cao and others [243] described how carrier-free immobilized enzymes generally exhibit 10-1000
times higher volumetric activity (U/g) \textit{versus} carrier-bound. Carrier-free immobilized CALB can also be used and recycled in both discontinuous [54] and continuous [244] processes for ester synthesis with excellent operational stability, even under harsh conditions (e.g. SC-CO$_2$ at 150 °C and 10 MPa) [241, 245]. The combination of these attributes along with the ease of recyclability (ability to easily separate using simple decanting and purify using distillation techniques due to their high boiling point) add to their growing application in diverse processes and make them useful alternatives to organic solvents.

In an effort to determine the optimal IL for CALB synthesis, Lozano and others [246] tested six different ILs. All the assayed ILs proved adequate media for enzyme-catalyzed transesterification, and activity was increased with respect to that obtained in organic solvents of similar polarity. For example, 1-ethyl-3-methylimidazolium tetrafluoroborate enhanced 5- and 4-times the synthetic activity and half-life time respectively of lipase in comparison to 1-butanol [246]. Lou and others [240] also found that The IL 1-butyl-3-methylimidazolium tetrafluoroborate can serve as an excellent co-solvent with buffer in place of traditional organic solvents for Novozym 435 mediated hydrolysis of d,l-phenylglycine methyl ester with the advantage of markedly improved enantioselectivity and activity.

ILs have been previously studied in combination with enzyme enhancement techniques such as immobilization, microwave heating, and biphasic media. Combing the use of ILs with different immobilizations resulted in increasing the half life of CALB up to six-fold in hexane at 95 °C and increased synthetic activity up to six-fold in SC-CO$_2$ [158]. Combining microwave heating with 20 different ILs with different polarity,
viscosities, hydrophobicity, and water content revealed that IL stabilization and activation effects on CALB were greatest with dried ILs and non-dried enzymes [247]. Using ILs in a bi-phasic media resulted in a stabilized CALB which allowed the enzyme to be reused after 7-10 cycles with optimum conditions yielding nearly 100% isoamyl acetate conversion [52]. Lastly, HHP stabilized and activated CALB (shown in chapter 2 and 3) in hexane. However, the effects of combining HHP and an IL-alcohol biphasic system on any enzyme catalyzed reaction have yet to be documented.

The working hypothesis of this study is that HHP in an IL-alcohol biphasic system can be used to increase the rate of free and immobilized lipase catalyzed isoamyl acetate synthesis. The specific objectives are: 1) determine the production of isoamyl acetate from isoamyl alcohol and acetic acid by free and immobilized lipase in an IL-alcoholic biphasic system, 2) determine the effects of high pressure on the activation energy of free lipase in an IL-alcoholic biphasic system, and 3) determine the effect of temperature on the activation volume of free lipase in an IL-alcoholic biphasic system.

**Materials and Methods**

**Materials**

**Enzyme and reagents**

Lipases were from *Candida antarctica* lipase B (CALB) expressed in *Aspergillus oryzae* and either immobilized on a macro porous acrylic resin (Novozyme 435® E.C. 3.1.1.3: 13.10 U/mg) or free (BioChemika # 65986: 1.51 U/mg) and obtained from Sigma Aldrich (St. Louis, MO USA). Isoamyl alcohol, glacial acetic acid, hexane, hexanol, toluene, and the ionic liquid 1-Butyl-3-methylimidazolium hexafluoro-phosphate (bmimPF6) were obtained from Fisher Scientific (Pittsburg, PA USA). All solvents and substrates were held at -10 °C or on ice while preparing for assay.
HHP system

The HHP system was from Unipress Equipment (Warsaw, Poland) and all other auxiliary equipment and instrumentation was described in Chapter 2.

Methods

Reaction conditions

Enzyme was added (3.75 U/mL) into the reaction vial before adding isoamyl alcohol (76.5% v/v), ionic liquid (bmimPF6, 13.0% v/v), glacial acetic acid (9.45% v/v), toluene (as a internal standard, 0.39% v/v) and water (0.88% v/v), according to the optimized proportions presented by Feher and others [52] for the same system. The custom made reaction vial is depicted in Fig. 4-1and shown in Appendix B. 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 5 °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 the set-point. Upon completion of incubation, the temperature was returned to 5 °C, and the reactor was depressurized and opened. Adiabatic heating or cooling effects associated with HHP systems upon pressurization and depressurization were not significant factors because temperature controlled jacket limited temperature fluctuations to less than 2 °C. Pressure and temperature had no effect on the reaction in the absence of enzyme with or without presence of IL at pressures up to 400 MPa and 80 °C for 2.5 h (data not shown). Which was similar to previous studies [52, 224] where isoamyl acetate production was negligible in absence of a catalyst. Samples were treated and assayed in a randomized block design, blocked by temperature for
convenience, due to the time required to heat and cool water baths while pressure selected randmoly.

**Quantitative analysis**

At selected incubation times, the reactor was cooled, depressurized and the reaction vial was withdrawn. A 10-μL aliquot was drawn from the top (alcohol) phase through the Luer-lock™ tip, diluted to 1 mL in hexane, and dried over excess ammonium sulfate anhydrous and sodium bicarbonate to remove remaining acetic acid and water. Then 10 μL of $7.9 \times 10^{-5}$ mM hexanol in hexane was added as a second internal standard in preparation for GC-FID analysis. The toluene standard was added at the initiation of the reaction to account for error associated with transferring and sample loss, while the hexanol internal standard added prior to GC-FID injection accounts for error associated with the GC-FID analysis. Reaction progress was monitored using GC-FID 5890 (HP, Palo Alto, CA USA) with a ZB-5 column (30 m length × 0.53 mm ID × 1.5 μm thickness) at a gradient temperature from 50 °C to 90 °C at 5 °C min⁻¹. Injector temperature was held at 200 °C and FID detector at 250 °C. Peak identification and quantification was determined using pure standards and a calibration curve. Linear regression analysis was conducted to generate apparent initial reaction rates expressed as rate of isoamyl acetate formed per enzyme unit. Reaction stoichiometry has been recently reviewed and confirmed [38, 42, 221] as shown in equation 2-1, which allowed kinetic analysis to be conducted by following product (isoamyl acetate) formation.

**Conversion using free and immobilized lipase**

Free or immobilized lipase was incubated at either 0.1 or 300 MPa for 3 h at 42, 63.5, or 80 °C. After incubation was complete the reaction vial was withdrawn and an
aliquot was analyzed as previously described. Reactions and analysis were conducted in triplicate and error represented as standard deviation.

**Activation energy**

Free lipase was incubated at either 0.1 or 300 MPa at 40.0, 49.4, 59.0, 69.3, or 80.0 °C. At 30-min intervals the reaction mixture was cooled, depressurized, and aliquots were taken and analyzed as described above to determine apparent initial rate. Linear regression analysis was conducted using Excel (Microsoft Office 2007) data analysis tool. Error was represented as standard error of the linear regression. Apparent activation energy ($E_a$) was determined using the Arrhenius equation (shown in Equation 1-7, Chapter 1).

**Activation volume**

Free lipase was incubated at pressures from 0.1 to 500 MPa at 40 or 80 °C. At 30-min intervals the reaction mixture was cooled, depressurized, and aliquots were taken and analyzed as described above to determine apparent initial rate. Linear regression analysis was conducted using Excel (Microsoft Office 2007) data analysis. Error is represented as standard error of the linear regression. Activation volume ($\Delta V^\ddagger$) was determined using Eyring equation (described in Equation 1-1, Chapter 1).

**Ionic liquid recovery**

After each reaction cycle, the biphasic mixture was decanted to separate the alcoholic and IL phase containing the lipase. The lipase and ionic liquid was then filtered through filter paper (type: P8) from Fisher Scientific (Pittsburg, PA USA) to remove protein aggregates. Then, to remove trace volatiles the IL was held at 70 °C under vacuum for 8 h.
Results and Discussion

Qualitative Observations

The biphasic system appeared to change depending on the incubation conditions. As shown in Fig. 4-2A the native enzyme-IL mixture appears clear with yellow suspended enzyme. As shown in Fig. 4-2B, after incubation at 0.1 MPa the enzyme appears white and mostly accumulated at the IL-alcohol interface. Following incubations between 100 and 300 MPa (Fig. 4-2C), the enzyme appeared to be in a dispersed cloud above the IL-alcohol interface. After incubation at ≥400 MPa the enzyme-IL mixture appeared to be a semi-solid white mass upon depressurization and cooling (shown in Fig. 4-2D). After approximately 15 minutes at ambient pressure and temperature all treatments appeared as Fig. 4-2B. The formation of the white mass was also observed with just the pure ionic liquid (data not shown). It remains unclear when this apparent phase change occurs; either during come-up, incubation, or come-down. Further analysis utilizing a high pressure optical cell is needed to determine accurately the conditions which induce this previously unseen phenomenon. This temporary immobilization shown in Fig. 4-2D has not been previously described, and may be useful in aiding the separation of phases between reaction cycles.

Conversion with Free and Immobilized Lipase

Increasing temperature increased the production of isoamyl acetate for both free and immobilized lipase (which is within the linear range of conversion as shown in Fig 4-3) as shown in Fig. 4-4 and Fig. 4-5. Production also increased at 300 MPa compared to at 0.1 MPa reaching a maximum relative increase of 4.9-fold at 63.5 °C with free lipase. Production was ~10-fold higher with free lipase compared to immobilized lipase. Based on these findings, the rest of the experiments used free lipase instead of
immobilized lipase due to catalytic efficiency differences. Production may be higher with free lipase due to increased substrate-active site collisions associated with free lipases becoming more dispersed in the alcohol phase due to continuous stirring as discussed below.

Conversion of isoamyl alcohol and acetic acid to isoamyl acetate was more efficient in an IL-alcohol biphasic system than in the mono-phasic system used in Chapter 2. The maximal concentration after 95% conversion was about 60 mM after 1 h in hexane, while the maximal concentration after 95% conversion was about 1500 mM after 4 h in the IL-alcohol biphasic system. Furthermore, catalysis in an IL-alcohol biphasic system benefits from the advantage of potentially having an inhibitory substrate, acetic acid, in a different phase than the enzyme, thus reducing substrate inhibition [52]. For example, acetic acid was shown to have strong inhibitory effects on lipase at concentrations as low as 0.1 M in hexane (increasing concentration from 0 to 0.1 M doubled the time required to reach full conversion) [221]. High concentration of acetic acid may be affecting the pH of the aqueous mono-layer around the enzyme. However, acetic acid concentration can be increased up to 3.6 M in a “solvent free” (isoamyl alcohol based solvent) system before experiencing lipase inactivation [47]. Therefore, utilization of the IL-alcohol biphasic system benefits from the lipase being in a separate phase than the acetic acid and from the increased allowable concentrations of acetic acid due to being in the alcohol based “solvent free” system.

**Determination of Activation Energy**

Temperature effects on apparent initial rate are shown in Fig. 4-6 and transformed into Arrhenius plots in Fig. 4-7. The apparent activation energy \( (E_a) \) derived from the slope of the lines in Fig 4-7 is shown in Table 4-1. It is assumed that apparent initial
rates are representative of the actual initial rates. It is also assumed that the substrate (acetic acid) is available to CALB in excess ($K_M << [S]$). Although the $K_M$ of this reaction has not been determined in this IL-biphasic mixture, it has been determined in monophasic hexane in Chapter 3 to be from 2 to 38 mM (acetic acid) depending on the reaction conditions. This reaction in the IL-alcoholic biphasic system utilized acetic acid at 1589.5 mM which is much greater than the $K_M$ determined in hexane. Pressure only affected the $E_a$ of immobilized lipase (28% increase) which was similar to isoamyl acetate synthesis catalyzed by CALB at HHP in hexane as discussed previously in Chapters 2 and 3. The $E_a$ values in Table 4-1 are higher than similar previous studies in an IL-biphasic mixture (27.3 kJ mol$^{-1}$) [52], in a solvent free system (28.7 kJ mol$^{-1}$) [47], and in hexane (35.7 to 47.8 kJ mol$^{-1}$) at various pressures (from Chapter 2). With regards to $E_a$ in IL-biphasic mixture, differences may be attributed to use of different temperatures, since this study examined 5 temperatures from 40 and 80 °C while Feher and others [52] used only 4 temperatures between 30 and 60 °C with no mention of the error. Likewise, Guvenc and other [47] only used 3 temperatures from 30 to 50 °C to determine $E_a$ with no mention of error. Finally, the $E_a$ results from Chapter 2 in hexane used the same lipase but it was immobilized, which may account for differences in $E_a$. Unlike previous results in hexane (from Chapter 2), this study in IL-alcohol biphasic system found a continual increase in reaction rates within the temperature range tested (up to 80 °C) at all pressures tested. This may be attributed to the stabilization effects of ionic liquid and/or high pressure on lipase. The possible mechanisms of pressure induced stabilization have been previously described in Chapter 1 and 2. Although the stabilization effects of ILs has yet to be fully understood, it is thought to be related to the
ILs ability to 1) negate substrate or product inhibitory action, 2) prevent essential water loss associated with thermal denaturation as organic solvents do, 3) remove excess water produced from esterification reactions also as organic solvents do, and 4) have electrostatic interactions with the enzyme. Furthermore, Lozano and others [54] suggested that since ILs form a strong ionic matrix, and added enzyme is considered dispersed or included but not dissolved, an optimal microenvironment may be formed for improved stability and activity. This last proposed mechanism is the most likely since organic solvents also have interactions with substrates and/or products, restrict essential water loss, facilitate excess water removal, and can have electrostatic interactions with the enzyme but do not possess the ability to disperse an enzyme like an IL. However, the activities are different when using the same enzyme and the same reaction but changing the solvent system.

**Determination of Activation Volume**

As shown in Fig. 4-8, increasing pressure increased the apparent initial reaction rate of free lipase at 40 and 80 °C from 0.1 to 500 MPa. It is assumed that apparent initial rates are representative of the actual initial rates. It is also assumed that the substrate (acetic acid) is available to CALB in excess ($K_m << [S]$) for the reasons previously discussed when describing the activation energy results. This reaction utilized acetic acid at 1589.5 mM which is much greater than the $K_m$ determined in hexane. Pressure effects resulted in a 15 and 25-fold increase in apparent initial rate from 0.1 MPa to 500 MPa at 40 and 80 °C respectively. The activation volume ($\Delta V^\ddagger$) was $-16.1 \pm 1.5$ and $16.7 \pm 1.4 \, \text{cm}^3 \, \text{mol}^{-1}$ at 40 and 80 °C respectively and was derived from the slope of the plots shown in Figure 4-9. The $\Delta V^\ddagger$ was negative and unchanged by temperature, therefore increasing pressure continuously increased initial rates.
regardless of temperature. These results are different than in chapter 3 where the negative $\Delta V^\ddagger$ of CALB catalyzed isoamyl acetate synthesis in hexane was decreased by increasing temperature from $-21.6 \pm 2.9$ at 40 °C to $12.9 \pm 1.7$ at 80 °C. This study found a negative $\Delta V^\ddagger$ throughout the entire pressure range tested (0.1 to 500 MPa). This is in contrast to CALB in hexane from chapter 2 that found a negative $\Delta V^\ddagger$ from 0.1 to 200 MPa (-21.6 and -12.9 at 40 and 80 °C respectively) and positive $\Delta V^\ddagger$ from 300 to 500 MPa (14.3 and 15.2 at 40 and 80 °C respectively). These differences between temperature affects on $\Delta V^\ddagger$ indicate that the solvent (either hexane or IL) has an effect on pressure induced activation.

Similarly, the activity and selectivity of free Candida antarctica lipase B (CALB)-catalyzed trans-esterification of butyl butanoate (a flavor ester) were both higher in water-immiscible but not water-miscible ionic liquids than in hexane [248]. This was explained as being due to the increasing hydrophobicity of the IL involves a better preservation of the essential water layer, reducing the direct protein-ion interactions. Likewise, CALB increased activity in all four ILs tested in comparison with hexane and 1-butanol. Also, according to Lozano and others [54], the lipase was “over-stabilized” in ionic liquids as evidenced when the reuse of free lipase in continuous operation cycles showed a half-life time 2,300 times greater than that when the enzyme was incubated in the absence of substrate, and had selectivity higher than 90%. Interestingly, IL pretreated lipase also had higher activity and stability than untreated lipase for the hydrolysis of $p$-nitrophenol butyrate in phosphate buffer. According to Nara and others [238], pretreated lipase maintained activity after seven days at 60 °C in phosphate buffer, while untreated lipase was fully inactivated after only 12 h at the same
conditions. It was suggested that this may be caused by the IL-coated lipase having altered structure thus enhancing activity and stability of the lipase. More recently, Zhao studied [247] microwave heating effects on CALB activity in twenty different ILs. Higher activity was observed compared to conventional thermal heating and varied depending on IL purity, polarity, hydrophobicity, and viscosity.

Besides conventional organic solvents or IL-alcohol biphasic mixtures, compressed or supercritical fluids/gases have also been examined as solvents at elevated pressures. CALB was shown to be effective at synthesizing isoamyl acetate in SC-CO$_2$ at higher initial reaction rates when compared to n-hexane while maintaining activity from 8 to 30 MPa [161]. These results were explained as being due to increased diffusivity of the reactants in the medium. In a similar study at 60 °C and 8 MPa in SC-CO$_2$, lipase activity increased 84-fold with respect to synthesis in organic solvents while maintaining stability showing a 360 cycle half-life. Increased activity was explained as improved micro-environments around the enzyme [108]. In another study, Pseudomonas cepacia lipase (PCL) catalyzed transesterification of 1-phenylethanol with vinyl acetate in SC-CO$_2$ showed an increase in catalytic efficiency up to 15 MPa. However, $\Delta V^\#$ did vary with pressure range used, reaching a maximum negative value of -1340 cm$^3$ mol$^{-1}$ at 7.4 MPa [55]. High negative $\Delta V^\#$ would be expected in SC-CO$_2$ 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.
The combination of IL and HHP may be altering the tertiary structure of the lipase, thus activating and/or stabilizing the lipase. Stabilization allows the activation effects to be observed at a wider temperature-pressure range.

**Conclusions**

This study has provided evidence that the conversion of isoamyl alcohol and acetic acid to isoamyl acetate in an IL-alcoholic biphasic system is 10 times more efficient with free than with immobilized lipase. Also rate of conversion was higher at elevated pressure for both free and immobilized lipase. High pressure had no effect on the apparent activation energy of free lipase and temperature had no effect on the apparent activation volume, which was negative throughout the pressure range tested. Lastly, the observation of a temporary immobilization of free lipase within the IL phase provides new insight into a possible technique, such as rapid decanting, for improving separation of the reactants and products from the IL-enzyme mixture.

**Acknowledgements**

This project was supported by a Research Innovation Grant from the University of Florida Institute of Food and Agricultural Sciences.
Figure 4-1. Depiction of the custom made IL-HHP reaction vial.

Figure 4-2. Reaction vessels with ionic liquid biphasic mixture and free lipase.
Figure 4-3. Concentration (mM) of isoamyl acetate produced by CALB (7.5 U/ml) in an IL-alcoholic biphasic system at 40 °C. Error bars represent standard deviation of three replicates.

Figure 4-4. Concentration (mM) of isoamyl acetate produced after 3 h incubation per unit enzyme of free CALB at (grey) 0.1 or (black) 300 MPa. Error bars represent standard deviation of three replicates.
Figure 4-5. Concentration (mM) of isoamyl acetate produced after 3 h incubation per unit enzyme of immobilized CALB at (grey) 0.1 or (black) 300 MPa. Error bars represent standard deviation of three replicates.

Figure 4-6. Apparent initial rate per unit enzyme of free CALB at (○) 0.1 or (□) 300 MPa. Error bars represent standard error of linear regression.
Figure 4-7. Determination of activation energy using the Arrhenius equation at (◊) 0.1 and (□) 300 MPa.

Figure 4-8. Apparent initial rate per unit enzyme of free CALB at (○) 40 or (□) 80 °C. Error bars represent standard error of linear regression of five data points.
Figure 4-9. Determination of activation volume using the Eyring equation at (◊) 40 and (□) 80 °C.

Table 4-1. Activation energy ($E_a$) of free and immobilized lipase at 0.1 or 300 MPa

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>$E_a$ ± Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>43.4 ± 3.1</td>
</tr>
<tr>
<td>300</td>
<td>55.4 ± 0.1</td>
</tr>
<tr>
<td>Free</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>55.6 ± 4.2</td>
</tr>
<tr>
<td>300</td>
<td>56.2 ± 4.6</td>
</tr>
</tbody>
</table>
CHAPTER 5
IN-SITU ANALYSIS OF LIPASE STRUCTURAL CHANGES INDUCED BY HIGH PRESSURE AND THERMAL TREATMENT

Introduction

High pressure (HP) affects proteins and enzymes by changing their structural conformation which can be monitored using spectroscopic techniques. As discussed in the previous chapters, pressure induced activation and stabilization may be related to conformational changes in the enzyme structure. Currently, the effects of (HP) on enzyme structure is scarce because there are few laboratories equipped with HP optical cells coupled to the instrumentation necessary for such studies. Most conventional methods of monitoring conformational changes in enzyme structure induced by HP require ex situ measurements. While these studies are adequate for certain studies, they provide no insight into the continuous conformational changes that occur during each sequence of heating, pressurization, hold time, cooling, and depressurization. Lastly, the protein/enzyme may be reversibly denatured depending on the conditions; therefore observations conducted after a treatment at ambient conditions would be less than accurate.

Some conformational studies have been conducted in situ at elevated pressure using various other technique such as; Raman spectroscopy [201, 249], Fourier transmission [250], nuclear magnetic resonance (NMR) [251], X-ray scattering [228], and second and fourth ultraviolet derivative spectroscopies [227, 252-255] have also been developed for protein and/or enzyme analysis at HHP.

The objective of this study is to continuously measure in situ conformational changes in lipase at HP. Achieving this objective will allow greater understanding of
conformational changes at elevated pressures which may be correlated to pressure induced activation and stabilization.

**Materials and Methods**

**Materials**

**Enzyme and reagents**

Free lipase was from *Candida antarctica* lipase B (CALB) expressed in *Aspergillus oryzae* and (BioChemika # 65986: 1.51 U/g) and obtained from Sigma Aldrich (St. Louis, MO USA). Lipase was dissolved in a 20 mM sodium phosphate-dibasic buffer pH 7 at a concentration of 20 U/mL and stored at 5 °C. Polydimethylsiloxane silicone liquid (Accumetric, Inc., Elizabethtown, KY USA) was added as hydraulic fluid to fill the high pressure reactor.

**Fiber optic spectrometer**

Spectral analysis is achieved with a USB 2.0 Fiber Optic Spectrometer (Model USB4000) controlled and monitored by OOIBase 32 spectrometer operating software and connected by 600 μm core diameter fibers (model QBIF600-VIS/NIR) all from Ocean Optics (Dunedin, Fl). The UV-Vis-near IR light source is a Deuterium-Halogen light (model DH-2000) from Mikropack (Ostfildern Germany).

**High pressure optical cell**

Determination of structural changes in lipase at pressures up to 500 MPa is achieved through use of a custom designed and manufactured HP cell. The HP optical cell and connections is depicted in Fig 5-1 and a picture is shown in Appendix C. It has 3 sapphire optical windows at 90 and 180° angles which allow monitoring of a small sample (~2 mL) though fluorescence spectroscopy. The HHP optical cell is pressurized and temperature controlled by a HHP micropump (model MP5), and a pump controller
(MP5 micropump control unit) all from Unipress Equipment (Warsaw, Poland). The optical cell is temperature-controlled with a water jacket alternatively fed by two water baths (Isotemp 3016D); one cooling (10 °C ± 0.1 °C) and another heating (80 °C ± 0.1 °C) from Fisher Scientific and controlled by an array of pinch valves. The optical HHP cell is comprised of stainless steel and CuBe alloy and has connections for thermocouples.

**In-Situ optical analysis**

Fluorescence emission spectra is recorded continuously *in situ* with an excitation of 285 nm and scanned at a right angle in the emission range of 250-500 nm while recording at 350nm (the specific wavelength of tryptophan) according to a previous study [169]. Changes in tryptophan residues are observed as they shift from buried inside the enzyme (native) to exposed on the surface (denatured) or *vice versa*. Changes in fluorescence of buffer as well as pressurization fluid are negligible as determined by monitoring fluorescence changes in the absence of enzyme. A continuous flow of nitrogen blanketed the sapphire windows to prevent moisture condensation upon cooling. After insertion of the sample cuvette, the reactor was sealed and pressurized. After pressure reached 500 MPa, temperature was adjusted to 80 °C using the pressure-first method discussed in Chapter 2. Upon completion of the incubation time, the reactor was cooled to 10 °C and depressurized to 0.1 MPa. This heating and pressurization sequence was repeated with continuous fluorescence monitoring to observe cycling effects. 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.
Results and Discussion

The results of continuous monitoring of fluorescence at 350 nm are shown in Fig. 5-2. Fluorescence intensity (indicative of tryptophan exposure) changed depending on the applied pressure and temperature combination as well as the number of previous cycles. Upon initial pressurization the fluorescence intensity decreases then recovers slightly after reaching 500 MPa. With increasing temperature the fluorescence intensity increased. During incubation the intensity remained constant or slightly decreased before decreasing during cooling. After depressurization the intensity decreases again to reach a value lower than prior to treatment. This general trend is repeated with each cycle although the magnitude of change is lessened. These results indicate that lipase conformation is altered depending on the temperature-pressure combination, which offers a possible explanation to pressure induced activation and/or stabilization. However, the scattering effects associated with enzyme denaturation and aggregations have not been accounted for.

In a similar study, the effects of incubating *Rhizomucor miehei* lipase at temperatures from 40 to 60 °C and 0.1 MPa or at pressures from 300 to 500 MPa at 25 °C was observed using fluorescence spectroscopy *ex situ* [169]. Results suggested that the conformational changes induced by pressure were different from those induced by temperature, which in agreement with the findings in Fig. 5-1. In contrast, no significant shift in the 350 nm band was observed for thermal treatment, which is different than found in this study.

According to the previous results discussed in Chapters 2 and 3 activity is higher at elevated pressure-temperature combinations and according to the results shown in Fig 5-1 tryptophan fluorescence is also increased at elevated temperature-pressure
combinations. Therefore, there may be a correlation between pressure induced activation and pressure induced conformational changes. This is in agreement with Noel and others [169] who found that the intensity of lipase tryptophan fluorescence decreased with a corresponding decrease in activity. In other words, a 63% decrease in relative fluorescence corresponded to an 80% reduction in relative residual activity. However, a direct comparison between results is difficult due to differences in lipase, pressure-temperature combinations, and measuring \textit{in situ versus ex situ}.

\textbf{Conclusions}

Fluorescence spectroscopy of lipase has been conducted continuously \textit{in situ} at pressures up to 500 MPa and up to 80 °C. Results indicate immediate and profound changes in tryptophan fluorescence as indicated by changes in the intensity at 350nm. Evidence has been provided for a possible link between pressure induced activation and pressure induced conformational changes. Although these results are qualitative, they do provide a valuable insight into the conditions which trigger conformational changes.

\textbf{Acknowledgements}

This project was supported by a Research Innovation Grant from the University of Florida Institute of Food and Agricultural Sciences
Figure 5-1. Schematic representation of the high pressure optical cell with sapphire windows and fiber optic ports for continuous optical measurements \textit{in situ}. 

Figure 5-2. Intensity expressed in counts of fluorescence at 350 nm emitted from lipase during 3 cycles of pressurization (0.1 to 500 MPa), heating (10 °C to 80 °C), and 2 minute incubations followed by cooling and depressurization.
CHAPTER 6
COMPREHENSIVE RESULTS, DISCUSSION, AND FUTURE STUDIES

This research has filled various gaps in knowledge. The first chapter provided
evidence that high pressure stabilization and activation is not an isolated phenomenon
and occurs in many different enzymes, from different sources, in different
solvents/media, and with different optimal conditions. These findings are important
because they provide a base of evidence which can be used to further explore the
application of high pressure to improve enzyme catalysis. Since most enzymes
discussed are used in the food industry, there remains a gap in knowledge with regards
to enzymes from other industries. This has yet to be explored because high pressure
processing is a relatively new technology which has not be widely implemented in other
production processes. To fill this gap in knowledge, a broad sampling of enzymes
should be conducted from other industries (i.e. pharmaceutical industry) and studied
with regards to their pressure induced stabilization or activation properties.

Also in the first chapter, the possible mechanisms of pressure induced stabilization
and activation were compiled and discussed. Although several studies have alluded to
possible mechanisms, this was the first to comprehensively describe them and the data
behind these proposed mechanisms. By fully understanding the current state of
knowledge, future studies can be executed to explore the validity of these proposed
mechanisms, which will allow for better utilization of pressure-temperature
combinations.

The second and third chapters provided evidence that HHP can stabilize and
activate (up to 152% and 239% respectively) an immobilized lipase used to catalyze the
synthesis of isoamyl acetate in hexane. The gap in knowledge was filled by
determination of several parameters; including $\Delta V^\ddagger$, $E_a$, $V_{max}$, and $K_M$ while also observing the effects of HHP on the immobilization support. This study was important because it was the first to demonstrate pressure induced enhancement of the most widely used enzyme (in synthesis reactions) in the most commonly used solvent (hexane) for the production of a widely used product (isoamyl acetate). As a model system, this study provides evidence of a new method to increase lipase activity and stability that can be implemented in combination with existing enhancement techniques such as immobilization, genetic engineered enzymes, and non-aqueous solvents. However, several important questions remain unanswered. They include; what are the mechanisms associated with pressure induced changes on reaction rate and substrate-enzyme binding, what is the effect of temperature on $\Delta V^\ddagger$ and $\Delta V K_M$, and what are the long term effects on lipase stability and activity caused by HHP and high temperature cycling. These questions remain unanswered due to lack of adequate time and proper equipment. To explore mechanisms, a high pressure optical cell coupled to an instrument capable of monitoring specific conformational changes (e.g. circular dichroism) is required. Furthermore, a lipase that is soluble in a non-aqueous and non-polar medium must be developed to adequately describe conformational changes associated with increased activity and stability in hexane. To determine the effect of temperature on $\Delta V^\ddagger$ and $\Delta V K_M$ a series of assays are needed at a range of temperatures and pressures. Lastly, cycling effects over time can be determined by repeated pressurizing, heating, cooling, and depressurizing until significant activity is lost. Cycling studies can be conducted more efficiently by using a multi-cell high pressure reactor. When these studies are complete and with the growing use of high
pressure processing driving the advancement of high pressure equipment and processing technologies, the industrial commercialization of this HHP technique will be a more feasible option for enzyme catalysis.

The fourth chapter demonstrated the activation effect (up to 25-fold activity increase) of HHP on immobilized and free lipase used to catalyze synthesis of isoamyl acetate in an ionic liquid-alcoholic biphasic mixture. The effects of HHP on lipase activity were unknown in an ionic liquid-alcoholic biphasic mixture as no studies had been done with any enzyme at HHP in this solvent system. This gap in knowledge was achieved by the determination of both $\Delta V^f$, $E_a$ of lipase catalyzed isoamyl acetate synthesis. These findings are important because they show how other solvent systems (besides mono-phasic organic) can also be used in combination with HHP. Furthermore, they demonstrated that free lipase is better suited than immobilized lipase in this system which will allow future studies to proceed more effectively. Lastly, the observation of the temporary immobilization phenomenon is a new and previously undocumented occurrence. This unique phenomenon may aid in recycling during a batch process by increasing the ease of decanting procedures while minimizing enzyme and IL loss. The remaining gaps in knowledge include; how HHP and thermal treatment cycling affect enzyme activity and stability, what is the best method of recycling the IL while ensuring complete removal of dispersed lipase, and when does the temporary immobilization phenomenon occur, how does it affect activity or stability, and how can it be utilized to improve the reaction processing/recycling in a batch or semi-continuous system. These questions remain unanswered due to time constraints, but can be readily answered using currently available methods and equipment.
Lastly, in the fifth chapter a relationship between pressure-temperature induced lipase activation and conformational changes was established. It was previously unknown if HHP induced activation and stabilization was directly related to conformational changes. These findings are important because they provide evidence that HHP has direct effects on the catalyst (lipase) aside from any effect on the chemical reaction, solvent, substrate, etc. With this evidence further studies can explore precisely what changes are occurring in the lipase that account for increased activity and stability. These needed studies were not conducted due to lack of proper spectral monitoring equipment.

Although this research has filled some gaps in knowledge, much remains unanswered. Further research is needed in using high pressure technology for more cost-effective synthesis reactions such as the production of high value pharmaceuticals. Cost-effectives can also be improved through the development of a continuous high pressure reactor. Also, the optimization of enzyme catalysis in environmentally friendly solvents such as ILs may reduce or eliminate the need for using toxic and flammable organic solvents. Furthermore, if conformational studies can determine specific structural changes that account for activation and stabilization then techniques such as site-directed mutagenesis and site-specific immobilization may be applied to generate similar conformational changes with similar activity and stability while at ambient pressure.
APPENDIX A
PICTURE OF HIGH HYDROSTATIC PRESSURE SYSTEM

Cold Water Bath  Hot Water Bath  Magnetic Stirrer

Pump Controller

High Pressure Pump

Reactor
APPENDIX B
PICTURE OF HIGH HYDROSTATIC PRESSURE IONIC LIQUID REACTION VIAL

- Luer-Lok™ Tip
- Isoamyl Alcohol
- Immobilized Lipase
- Ionic Liquid
- Micro Stir Bar
APPENDIX C
PICTURE OF HIGH HYDROSTATIC PRESSURE OPTICAL CELL
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Michael was born and raised in Grand Island Nebraska. After high-school he completed his B.S. in animal science and his M.S. in food science at Oklahoma State University in Stillwater Oklahoma. After working as a research specialist he began his Ph.D. in food science at the University of Florida. Michael has served as the IFT-Florida section executive student representative, as the captain of the UF college bowl team, as captain of the Dansico product development team, as a member of the UF IFTSA product development team, and is active in a variety of other extracurricular activities.

Michael’s research Interests include improving the application and understanding of enzyme catalysis through use of novel and innovative technologies with focus on utilization of high pressure. His research has recently gained much attention after being awarded 1st place in the biotechnology division poster competition at the 2008 IFT annual meeting & Expo and 3rd place at the 2009 International Commission of Agricultural and Biosystems Engineering International Technical Symposium in Potsdam Germany. Michael research has been, and will continue to be published and cited in renowned scholarly journals and discussed at technical conferences.

Upon completion of the requirements for a Ph.D. in food science at the University of Florida Michael intends to seek a position within the food industry to apply his current and developing skill set to improve the availability and nutritional quality of the world’s food supply.