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

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ABSTRACT

Lipases are important to high value product synthesis, modification, and enhancement. However, they are often unstable above 40 °C. While most current applications of high hydrostatic pressure (HHP) are for inactivating deleterious enzymes, there is evidence that HHP can stabilize and increase activity of some enzymes. This study examines the apparent kinetics of immobilized lipase-catalyzed synthesis of isoamyl acetate at HHP in hexane. HHP reduced thermal inactivation of lipase by up to 152% after 4 h at 80 °C and 400 MPa when compared to incubations at low pressure. No significant differences were found in activation energy (E_a) at different pressures, irrespectively of the pressurization and heating sequence, and were between 35.7 ± 3.5 and 47.8 ± 8.2 kJ mol^{-1}, depending on the method. In all methods utilized, activity at 63.5 and 80 °C at 400 MPa was greater (from about 20 to 96% increase) than at low pressure. Activity increased by 110% at low pressure versus a 239% increase at 350 MPa when the temperature was increased from 40 to 80 °C. Increasing pressure up to 350 MPa increased lipase activity while pressures greater than 350 MPa maintained or decreased lipase activity. Activation volume ($\Delta V^{\neq}$) appeared negative between ambient pressure and 200 MPa in contrast to a positive $\Delta V^{\neq}$ between 300 and 600 MPa. Apparent $\Delta V^{\neq}$ was 14.3 ± 1.7 or 15.2 ± 2.2 cm^3 mol^{-1} at 40 or 80 °C, respectively, between 300 and 500 MPa.

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1. Introduction

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

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

Pressure and heat are generally thought to be antagonistic factors in molecular terms (from the principle of microscopic ordering, an increase in pressure at constant temperature leads to an ordering of molecules or a decrease in the entropy of the system) and with regards to enzyme conformation [21–23,35]. This antagonistic relationship results in an elliptical pressure versus temperature diagram with native and denatured protein regions inside and outside of the ellipse, respectively [21]. During the pressure and temperature come-up time the enzyme may undergo irreversible denaturation that results in a decrease in activity. While these antagonistic effects have been previously explored and discussed [21–23,35] no work to date has compared the different effects of applying HHP first then heating, heating first then applying HHP, or attempted to apply HHP and heat simultaneously and assessed the effects on rate constant and activation energy, in particular for lipase-catalyzed synthesis of isoamyl acetate in organic solvent.

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Operating at HHP offers other less complex advantages over conventional synthesis at ambient pressures. For example, *Candida antarctica* lipase B (CALB) is a relatively heat-tolerant enzyme that is active up to 100 °C [5]. Furthermore, several other extremely heat-tolerant (stable up to 150 °C) enzymes have been discovered and may have a potential application in biocatalysis in organic media [36–38]. However, the boiling point of most organic solvents is well below 100 °C. For example hexane boils at 68.7 °C (at 0.1 MPa) which restricts studies in hexane to be explored below that temperature. As pressure is raised the boiling point of organic solvents also increases allowing studies to examine activity above conventional temperatures at low pressure [39]. The ability to operate with organic or volatile solvents at temperatures above their conventional boiling point is a significant yet often overlooked advantage of HHP.

The cost of high pressure processing (HPP) has decreased over the last decade and become more widely implemented particularly in the food industry. In 2007 there were about 120 HPP industrial installations operating worldwide [40] with 80% of their equipment installed since 2000. HPP food has become a two billion dollar global market and is expected to comprise 450 million pounds/year in 2008 [41]. As demand for HPP equipment grows, innovation is expected to continue to reduce capital and operating costs [40]. Although HPP of bulk foods is currently more widespread, the much higher profit-margin sector of enzyme catalyzed flavor synthesis may have greater potential for adopting HPP.

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

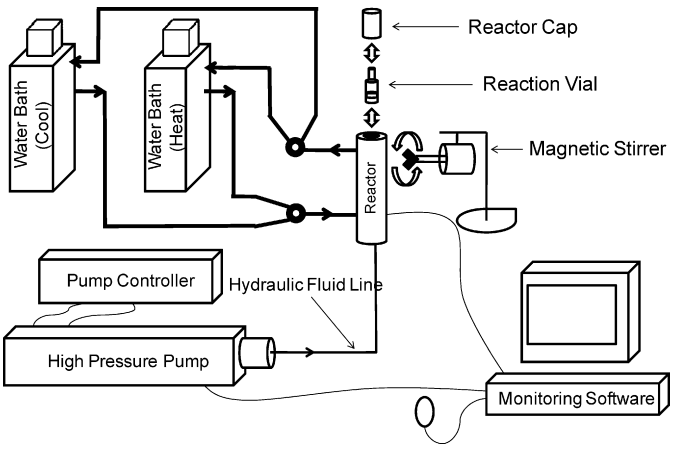


Fig. 1. Schematic representation of the high hydrostatic pressure system.

2. Experimental

2.1. Materials

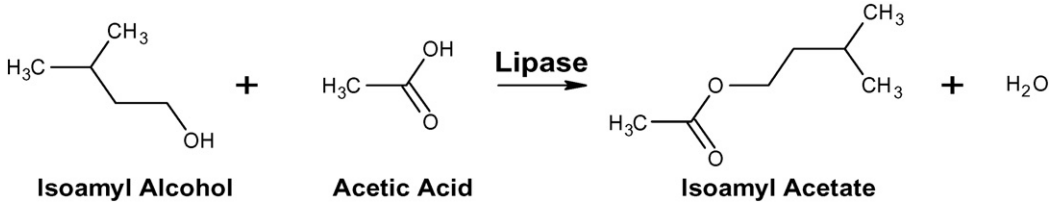
Lipase (Novozyme 435® E.C. 3.1.1.3) from *C. antarctica* lipase B (CALB) expressed in *Aspergillus oryzae* immobilized on a macroporous acrylic resin (13,100 PLU/g) was obtained from Sigma–Aldrich (St. Louis, MO USA). Isoamyl alcohol, glacial acetic acid, and HPLC grade hexane were obtained from Fisher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

The HHP system consisted of a high pressure reactor (model U111), a high pressure micropump (model 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 (25–80 ± 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. 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 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.

2.2. Methods

2.2.1. Effect of HHP on lipase stability

Enzyme was weighed (10 mg or ~128 Propyl Laureate Units) into the reaction vial. One milliliter of hexane and a miniature stir bar were added to the enzyme. The reaction vial plunger was moved into position to eliminate air bubbles then sealed with a 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 [3,5] and shown as



The esterification reaction was initiated by addition of 1 mL 0.12 M isoamyl alcohol in hexane and 1 mL of 0.12 M acetic acid in hexane combined to make 0.06 M substrate solution into the reaction vial through the Leur-lock™. The reaction mixture was incubated at 10 °C and stirred continuously. A 1-μL aliquot was drawn for GC-FID analysis every 10 min. The apparent initial rate was determined by linear regression of the progress curve in the linear range (time ≤30 min). Peak identification was determined using pure standards. Reaction stoichiometry has been recently reviewed and confirmed [3,5,7], which allowed kinetic analysis to be conducted by following product formation. Adiabatic heating or cooling effects commonly associated with HHP systems upon pressurization and depressurization were not significant factors as the temperature controlled jacket limited temperature fluctuations to less than 2 °C. Because the high pressure reactor was closed pressure increased simultaneously with temperature. When temperature reached 68.7 °C (hexane boiling point at ambient pressure) pressure had already reached 5 MPa. In other words, during the heating of the reaction cell, because of the simultaneous increase in pressure, hexane never reached the boiling point. Within the context of this study, pressures below 10 MPa, reached under these conditions, are referred as "low pressures". Pressure and temperature effects on the reaction, in the absence of enzyme, were ruled out when no significant reaction progress was observed at pressures up to 700 MPa and 80 °C for 4 h (data not shown) which was similar to previous studies [42] where isoamyl acetate production was negligible in absence of a catalyst. Native lipase activity was determined by assaying lipase that has not been exposed to temperatures above recommended storage conditions (5 °C). Percent relative activity was determined by comparing the initial rate of the observed reaction to that of the native enzyme (control). Samples were treated and assayed in a randomized block design, blocked by temperature and incubation time while pressure varied. Significant differences in treatments were determined using analysis of variance (ANOVA) and Tukey's pair-wise comparison at $\alpha = 0.05$ for enzyme treatments and controls. Statistical analysis was done using SAS software (Cary, NC, USA).

2.2.2. 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

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