HIGH HYDROSTATIC PRESSURE'S (HHP) EFFECT ON ENZYMATICSTABILITY

by

MICHAEL DIEHL

(Under the Direction of William Kerr)

ABSTRACT

High hydrostatic pressure (HHP) was applied to elucidate its effect on the thermostability of selected enzymes. Pressure reduced the thermal inactivation of lactate oxidase (LOx) at 66 °C and 0.1 – 50 MPa, ascorbate oxidase (AsOx) at 53 °C and 0.1 – 50 MPa, and cholesterol oxidase (ClOx) at 67.5 °C and 0.1 – 600 MPa. Pressure increased the rate of thermal inactivation of choline oxidase (ChOx) at 45 °C and 0.1 – 100 MPa. The optimal pressure for LOx and AsOx was between 50 MPa and 100 MPa based on the pressure where $\Delta V‡$ shifts from stabilizing to inactivating. Pressure stabilized up to 600 MPa for ClOx. In contrast, it is a denaturant for ChOx. For glucose oxidase (GOx) at atmospheric pressure, transition temperature (T') was 69.66 ± 0.45 °C and increased to 76.04 ± 0.15°C at 100 MPa. This positive shift of 6°C in $\Delta T'$ confirms pressure is preventing protein aggregation.

INDEX WORDS: High Hydrostatic Pressure, Enzymes, Stabilization

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CHAPTER 1

LITERATURE REVIEW

1. Introduction to High Hydrostatic Pressure

High hydrostatic pressure (HHP) has been extensively applied to foods to kill microorganisms and inactivate enzymes that catalyze reactions that negatively affect shelf life. The denaturing effect of HHP on proteins was first reported in 1914 for egg albumen at 500 – 700 MPa.[1] However, at pressures below 300 MPa, HHP can stabilize some enzymes against thermal denaturation.[2,3] During the last decades, interest in the effect of HHP on biotechnology has increased.[4,5] Several pressure effects on biological systems have been discussed and reviewed,[6] such as molecular interactions,[7] protein denaturation,[8] and protein stabilization.[9] Like temperature, a change in pressure can also affect the rate of enzyme catalysis. Therefore, HHP can alter in different ways an enzyme-catalyzed process. Even though the cost of HHP equipment has been decreasing as its application in the food industry continues to grow, it is only economically viable for high-added value products for niche markets and not for commodity products like most foods.[10] Therefore, industries with large profit margins such as the pharmaceutical, fine chemicals, medicine, and nutraceuticals industries can afford the cost of both enzymes and HHP technology.[11,12] HHP has also been used as a research tool for investigating the thermodynamics and kinetics of protein unfolding as well as of the biocatalytic reactions of enzymes.[9]

To be biologically active, enzymes must fold into unique three-dimensional conformations.[13] The specific amino acid sequence and folding of a protein most likely results

from the evolutionary response to the environment in which the organism that produces that enzyme lives. This explains why isozymes from different organisms have different amino acid sequences and quaternary structures, which result in different optimal conditions for stability and activity. The level of homology among isozymes does not always correlate with activity and stability. Indeed, a change in one single or very few amino acids can drastically change the enzyme stability, thermophilicity, and activity.[14] Small pockets or cavities of unfilled space that form in the process of folding correlate to some extent with the stability of the protein.[15] Furthermore, in some cases, HHP stabilizes folding intermediates. Such stabilization helps to elucidate misfolding events that lead to aggregation.[11] Uncovering and gaining a full understanding of the mechanisms of protein folding is one of the major challenges of modern-day science across multiple disciplines.[11]

After some theoretical considerations, this chapter reviews non-food applications of HHP on enzymes, food applications of HHP on enzymes, enzymes of interest, and the gap in knowledge to be addressed in the following chapter.

2. Theoretical Considerations

2.1 Effect of pressure on folding

Unfolding models are critical to hypothesizing and understanding the mechanisms of protein unfolding, which in turn are necessary to develop strategies. The simplest unfolding model considers two states: a denatured state, unfolded, and a native state folded. This two-state model is used to model the unfolding of single-domain proteins. Reversible unfolding from the native protein to the unfolded state is assumed:

$$N \leftrightarrow U$$

Where N is the native protein and U is the unfolded state.[16] However, unfolding is not always reversible. A protein can unfold and refold in a reversible fashion, but at times, aggregation and other interactions result in irreversible unfolding.[17] The thermal denaturation is often modeled by the Lumry-Eyring model. This model states that a reversible unfolding step is then followed by an irreversible denaturation step:

$$N \leftrightarrow U \rightarrow D$$

Where N is the native protein, U is the unfolded or partially unfolded protein, and D is the denatured protein.[18] Pressure affects protein folding by favoring the conformation that takes up the smaller molar volume. A completely unfolded protein that does not have any cavity would occupy the smallest volume. Thus, pressurization is often accompanied by a negative volume change.[19,20] However, one of the most commonly observed intermediates are molten globules. These intermediates retain their secondary structure but lack the tightly packed tertiary structure found in the native state. As a result, molten globules have a larger radius of gyration than their native counterparts.[21] Stabilization of the intermediate partially unfolded states helps to better understand the mechanisms of protein folding and unfolding. High pressure stabilizes these intermediates and coupled to spectroscopic techniques, have been able to characterize amyloidogenic proteins, prion protein, and lysozyme.[22,23]

Proteins spontaneously fold into their native state in which the structure has the minimum Gibbs free energy.[24] The Gibbs free energy for interprotein and intraprotein interactions depends on the volume of change (ΔV) of the reaction:

$$\Delta G\ (p) = \ \Delta G(0) + p\Delta V \tag{1}$$

$$\ln\left(\frac{a_p^n}{1-\alpha_p}\right) = p\left(\frac{\Delta V}{RT}\right) + \ln\left(\frac{K_{d0}}{n^n C^{(n-1)}}\right) \tag{2}$$

where at pressure p and at atmospheric pressure respectivily ΔG(p) and ΔG(0) represent the free energy of folding; volume of change ΔV; equilibrium constant for dissociation at atmospheric pressure K_{d0}; extent of the reaction α_p, and number of dissociating subunits n. Under pressure, proteins appear to favor a partially folded state.[11] Linear amino acid residues are packed together to form a three-dimensional structure. Due to the large amount of amino acids present in proteins and the multiple intramolecular interactions, folding can result in the formation of cavities in the protein. The core of the protein is a hydrophobic region that is, especially if cavities are present, the most susceptible area to pressure.[25-28] Most protein unfolding studies were traditionally done using high temperature, guanidine, urea, or other denaturants. These agents create changes that are unlikely to occur under biological conditions. Thus, high pressure has proved as a useful tool to investigate not only the packing of proteins but also their cavities.

2.2. Effect of pressure on enzyme kinetics

The application of pressure makes the equilibrium state's volume smaller and accelerates the volume change between the transition state and the ground state.[6] In other words, the pressure induces negative volume change and changes the activation energy during the catalytic reaction. At constant temperature, the change of activation volume during the catalytic reaction ($\Delta V^{\ddagger}$) can be defined by the transition state theory (Eq. 3), where P is the pressure, T is the temperature, R is the gas constant, and k_{cat}/K_m is the catalytic efficiency of the enzymatic reaction. Also, the change of activation volume during catalysis can be explained by the

difference between activated ($V^{\ddagger}$) and ground state (V_A).[29]

$$\Delta V^{\neq} = -RT\left[\frac{d \ln (k_{cat}/K_M)}{dP}\right]_T = V^{\ddagger} - V_A \quad (3)$$

The catalytic efficiency (k_{cat}/K_m) can be separated into activation volumes for substrate binding (ΔV_b) and catalysis (ΔV_{cat}) as shown in Eq 4. and Eq 5.[30,31]

$$\Delta V_b = -RT\left[\frac{d \ln K_M}{dP}\right]_T \quad (4)$$

$$\Delta V_{cat} = -RT\left[\frac{d \ln k_{cat}}{dP}\right]_T \quad (5)$$

The effect of HHP on enzyme kinetics is complex because HHP can also induce protein aggregation.[32] Furthermore, HHP can improve the thermal stability of some enzymes.[9,33-35] This stabilization effect allows for reactions to be carried out at temperatures higher than atmospheric pressure.

3. Non-Food Applications of HHP on Enzymes

An early interesting attempt to use HHP in combination with cooling for cryopreservation of organs did not result in significant advances in the preservation of tissue.[36] High pressure also caused the inactivation of tissue enzymes such as lactate dehydrogenase and glutamic oxalacetic transaminase activities above 70 MPa and 140 MPa respectively for treatments from -4 °C to -60 °C when pressure was applied before cooling. The medium composition and application of pressure after cooling also affected the rate of enzyme inactivation.[37] Recently, 50 MPa was applied to human dermal fibroblast cells for over 36 h to produce apoptotic inactivated cells. In vivo grafting of the treated cell did not cause the inflammation[38] produced by cells treated at 200 MPa that died by necrosis.[39] The effect of HHP on the enzymatic processes of cell death remains

unclear. To our knowledge, there is not yet any industrial application of HHP to the enzymatic production of drugs, vaccines, or nutraceuticals. However, two early excellent reviews from which we extracted some of the information in this section address the potential.[11,12]

3.1. Medical Research

High hydrostatic pressure has been applied in medicine, not as a treatment but as a tool for better understand folding intermediates, which is important when trying to understand protein aggregation and protein misfolding. Amyloidogenic diseases, including Alzheimer's, Huntington's, Parkinson's, emphysema, many types of cancers, spongiform encephalopathies, cystic fibrosis, and type II diabetes, are diseases that are caused by protein misfolding.[40-43] The partially folded and partially misfolded proteins lead to the formation of aggregates that are insoluble. In some cases, proteins undergo fibrillogenesis leading to the accumulation of fibrils. When these fibrils build up on specific tissues and in organs, it can lead to the death of the individual. A study using hen lysozyme showed that the formation of amyloid fibrillogensis is a two-step process in which revisable unfolding of monomers which is followed by the irreversible formation of amyloid fibrils.

$$U \Leftrightarrow U'_n \longrightarrow \text{fibril}$$

High pressure was able to dissociate the soluble assembly which prevents the formation of irreversible fibrils.[44] In Alzheimer's disease, amyloid β peptides and tau proteins undergo partial unfolding and form amyloid fibrils.[45] Dirix et al. reported the dissociation of early aggregates at relatively low pressure, but mature amyloid fibrils would not dissociate below over 1 GPa.[46] Conversely, HHP can induce the formation of fibrils from transthyretin (TTR).

Differences in the effect of HHP on the wild type and variants of TTR as well as α-synuclein, which are associated with Parkinson's disease, can help in the preliminary screening of effective drugs that prevent the formation of fibrils in vitro in a much shorter time than in vivo studies.[47]

3.2. Pharmaceuticals

Similar to applications in the food industry, HHP has been proposed for the sterilization of drugs whose activity would be affected by thermal or other sterilization approaches.[48] High pressure was also reported for the decellularization of implants for reduced rejection.[49] The development of vaccines against viruses and cancer HHP has also been proposed. These vaccines rely on the HHP-killing of viruses and the unfolding of proteins that inactivate the virulence or the cell ability to proliferate while retaining levels of immunogenicity higher than those produced by conventional methods.[50-53] Also, some pharmaceuticals have been successfully synthesized using enzyme-catalyzed reactions. However, there are only a handful of studies on the effect of HHP on pharmaceutical enzymes. Thermolysin can produce pharmacological peptides.[54] This enzymatic process is stereo and regio-specific.[55] An increase in pressure up to 200-250 MPa resulted in a 45-fold increase in the k_{cat}/K_m of thermolysin-catalyzed hydrolysis of Fua-Gly-Leu-NH_2.[56] However, longer incubations resulted in a decrease of the enzyme activity, indicating that, the optimal pressure for stability does not coincide with the optimal pressure for activity. Inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) through the inhibition of its precursor 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) can reduce cholesterol, thus decreasing the risk of cardiovascular diseases. Using peptides to produce such inhibition is an attractive alternative to other drugs that cause undesirable side-effects. Hydrolysates from a cocktail of lactic acid bacteria and *Spirulina platensis* produced by Umamizyme G, protease A,

or protease R were produced at atmospheric pressure or 100 MPa at 50 °C for 24 h. Hydrolyses carried out at 100 MPa produced greater peptide content and greater free amino acid content than hydrolyses carried out at 0.1 MPa. Hydrolysates produced by peptidase R at 100 MPa had the greatest inhibitory effect (67.0%) on HMG-CoA. These hydrolysates had greater peptide content and free amino acid content than all other treatments. Inhibitory peptides were Arg-Cys-Asp and Ser-Asn-Val.[57]

The practical application of HHP to industrially produce such hydrolysates or more specifically these inhibitory peptides would have to be analyzed from an economic perspective and compared to alternative means of synthesizing these peptides. Nevertheless, this research confirms that the mechanism of hydrolysis is affected by HHP. High pressure has also been used to increase the activity of peptidases from *Lactobacillus delbrueckii ssp.*[58] However, it is unclear whether the HHP treatment produced greater concentrations of the peptidases or peptidases with greater specific activity because the enzymes were not purified. While these peptidases were intended for use in the food industry, one can envision their use in other non-food applications. Lipase B from *Candida Antarctica* esterifies ibuprofen to produce a more soluble and bioavailable drug[59] but this reaction, to the best of our knowledge, has not been run using HHP. However, lipase activity and stability enhancement for other reactions have been reported.[60,61]

3.3. Nutraceuticals

Nutraceuticals, often extracted from foods and other plants, are dietary supplements that offer a number of health benefits, including potential disease prevention and therapeutic properties.[62,63]HHP can be applied to improve the activities of enzymes involved in the production of such phytochemicals, aiming to increase their yield. In addition, using high

pressure to stabilize heat-sensitive nutraceuticals increases the retention of potential anticarcinogenic properties.[12] For example, the hydrolysis of glucosynolates from cruciferous plants increased under pressure leading to the formation of isothiocyanates. As referenced in Masson et al.,[12] Sulforaphane, which has been studied for its antitumor activity and nematocidal properties,[64] can be converted from glucoraphanin found in broccoli juice. In addition, enzymatic hydrolysis for the production of bioactive peptides has been combined in sequence or simultaneously with HHP.[65] The pressure-induced partial unfolding of the protein increased by 23.9% the degree of hydrolysis of alcalase-catalyzed proteolysis kidney bean flour and by 30.1% the antioxidant activity of the resulting peptides.[66] Ginsenosides were extracted from ginseng roots at 100 MPa using a cocktail of cellulases, arabinose, xylanase, β-glucanase, and α-amylase with a 36.8% increase in yield relative to 0.1 MPa.[67]

3.4. Allergen reduction

Reduction of the risk of allergic reactions to foods is typically done by avoiding consumption or even entering in contact with such foods. Immuno-therapy may decrease the sensitivity to allergens. Removing the allergen through processing offers a potential alternative. High pressure treatment has been used to assist in the enzymatic hydrolysis of milk proteins, which has led to the reduction of allergenicity,[68,69] whereas the pressurization alone was found only to have a minimum effect.[70] In central Europe, a majority of adults have allergies caused by birch pollen allergen BET v 1.[71] Food allergens from various birch pollen plants, apple, carrot, celery, peach, cherry, and hazelnut, were tested by prick-to-pick test on allergic patients. A decrease in allergy symptoms was seen for some samples treated at 300 MPa and 600 MPa for 5 min. However, the decrease in allergenicity was not observed for all allergens. An in vivo and in vitro study was

conducted to investigate the effect of high pressure on the inactivation of allergen Mal d 1 from apple. In the study, none of the patients in the clinical tests experienced an increase in the severity of allergic reactions to the pressure-treated apples than the non-pressure treated apples. The author suggests that there are two mechanisms involved in the inactivation of the allergens: (i) volume compression altering the protein structure, which was verified via spectroscopy and in vitro tests and (ii) the cell disruption induced by high pressure enabled endogenous enzymatic process. During immunotherapy, a patient is exposed to a specific allergen in small doses causing the patients' immune system to trigger and induce tolerance. One hyposensitization strategy is to use a high-pressure treated allergen. This method was studied on patients with severe apple allergies. After being treated with 25 g of high pressure-treated apples every day for three weeks, the patients lost their allergy and were able to tolerate 50 g of untreated apple.[72]

1.4 Enzyme Applications in the Food Industry

4.1 Overview of Enzymes as Biocatalyst in the Food Industry

The United Nations Department of Economic and Social Affairs (UNDESA) has predicted that the population of the world will grow from 6.9 billion to 9.1 billion by the year 2050. This increase in pollution is expected to increase food demand by 70% over that same period. The main challenge will not be to grow 70% more food but making sure 70% more food is able to make it to consumers plates.[73] Food supply issues can be addressed using enzymes in the food industry. Enzymes have the ability to improve food production and components, including nutritional value, texture, appearance, aroma, flavor, and color.[74] The application of enzyme biocatalysts has increased across many industries not just the food industry, led by advancements in industrial microbiology and biochemical engineering increasing enzyme

applications. The global industrial enzyme market had an estimated valuation of $1.5 billion in 2000 and has grown steadily since, expected to reach valuation of over $6.3 billion by 2022.[75,76] Over half of the enzymes used for industrial purposes around the globe are used for food applications. The increased industrial application of enzymes within the food industry is one of the main growth factors in the market.[77]

In the food industry, enzymes are used as process aids and food additives across almost every sector of the industry, including beverages, dairy, brewing, fats and oils, meats, baking, and functional foods.[78] While each enzyme is highly selective, its applications can cross multiple sectors. The same enzyme can be used in different manufacturing processes and have different results. Glucose oxidase, when used by the baking industry, is able to make stronger dough.[79] Glucose oxidase can remove the glucose from egg white before spray drying to stop browning due to caramel formation.[80] Glucose oxidase is also used in food packaging to remove oxygen when in the presence of glucose, helping to improve shelf life.[81] Other enzymes and their applications in the food industry has been reviewed.[82]

Enzymes do have their limitations including low activity, poor stability, limited substates, high cost of isolation and purification, and limited usability outside of physiological window. Not all the problems have been solved, leaving an abundance of opportunity for future research.[74,82,83] The Reyes lab has been working on improving the stability and activity of a series of enzymes. These enzymes are oxidoreductase enzymes. Their reactions involve hydride transfer, insertion of oxygen, extraction of protons, or other essential steps.[84] Oxidoreductase enzymes commonly use the cofactors FAD, NADP, and NAD.[85] The series of enzymes include glucose oxidase (GOx),[86-88] alcohol oxidase (AOx),[33,89] xanthine oxidase (XOx),[90] and pyruvate

oxidase (POx).[91] Glucose oxidase[92-94] has applications in the food industry as a biocatalyst, while alcohol oxidase, xanthine oxidase, and pyruvate oxidase do not. Due to the highly selective nature of the enzymes, all of these oxidases can be used in enzyme biosensors that the food industry can use.[95] Due to stability and cost issues, glucose oxidase is the only one used commercially.[96]

4.2 Overview of Enzymes Used in Enzymatic Biosensors

In recent years there has been a dramatic increase in both the political and social awareness for current agricultural and industrial processes. Consumers and politicians are demanding the restriction of chemicals and biological species found in environmental, foodstuff and industrial products. In the food industry this has led the United States Food and Drug Administration (FDA), European Union Council, the World Health Organization (WHO), and the Food and Agriculture Organization (FAO) of the United Nations, as well as other national authorities to pass rules and directives accordingly. This has led to a high demand for efficient analytical methods to analyze the complex matrices and deliver fast and accurate results.[97] The typical methods are often highly complex, requiring access to a laboratory setting and quite lengthy. These methods for testing samples for analytes involve an electrophoretic or chromatographic separation process followed by electrochemical or spectrophotometric detection. The use of biosensors is one way to overcome these challenges. The advantage to using a biosensor is that it is low cost, highly specific, highly selective, and, in some cases, can be made portable to allow use outside of the laboratory in the field.[98]

A biosensor is an analytical device that uses a biological or biologically derived element, a transducer, and an electronic indicator to measure or quantify information. The biological

element can differentiate the specifically targeted molecules wanted to be tested. These biological elements can vary from receptors, antibodies, nucleic acids, microorganisms, lectins, and enzymes. The transducer creates a measurable signal from the biorecognition event. There are five common transducer classes optical, magnetic, thermometric, piezoelectric, and electrochemical. This signal is than processed by the electronic indicator to a readable form.[96] The first biosensor was invented in 1956 by Leland C. Clark to detect oxygen. The first commercially available one was a glucose biosensor in 1975 developed by Yellow Springs Instruments. Biosensors are now ubiquitous in medicine, food, pharmacology, forensics, and biomedical research. The two most popular biosensors today are pregnancy tests and glucose monitoring sensors.[99] The global biosensor market is expected to increase from $25 billion in 2020, with a compound annual growth of 7.4% during 2021 – 2027.[100] In the food industry, biosensors are used for food security, analysis of food composition, and process controls. The use of commercial biosensors has been extensively reviewed.[101]

There are few biosensors commercially available compared to the large amount of published research articles. The low stability and poor stability of the enzyme remains one of the greatest challenges facing biosensors for use for continuous monitoring of analytes. Commonly used methods for stabilizing enzymes are immobilization and high hydrostatic pressure.[102]

5. Enzymes of Interest

5.1 Choline Oxidase

Choline oxidase (ChOx) from *Alcaligenes sp.* is a dimer with a molecular weight of 120 kDa.[103] ChOx catalyzes the four-electron oxidation of choline to the intermediate betaine-

aldehyde to glycine-betaine via a two-step reaction. [104-106] Choline oxidase can be used in a biosensor to determine choline in powdered milk meant for children[107] and is a potential biomarker for early diagnosis of multiple cancers and neurological disorders, but enzyme stability holds ChOx biosensors back from commercialization.[108]

1.5.2 Cholesterol Oxidase

Cholesterol oxidase (ClOx) from *Streptomyces* is a monomer[109] with a molecular weight of 62 kDa.[110] ClOx catalyzes the first step of cholesterol catabolism in which cholesterol is oxidized to cholest-5-en-3-one requiring FAD, which then isomerizes to cholest-4-en-3-one with the release of hydrogen peroxide. ClOx is used in the medical industry to monitor human cholesterol levels, which can help reduce the risk of cardiovascular diseases. [111] There are also applications to be used in the food industry to measure the total cholesterol in foods like eggs, meat, and milk samples.[112-114]

1.5.3 Ascorbate Oxidase

Ascorbate oxidase (AsOx) from *Cucurbita sp* is a dimer with a molecular weight of 140 kDa.[115] AsOx catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. AsOx catalyzes a four-electron reduction of dioxygen to water accompanied by a one-electron of the reducing organic substrate**[116].** AsOx is used in the food and pharmaceutical industries to determine ascorbic acid (vitamin C) concentration in samples. Ascorbic acid is used heavily in the food industry as an antioxidant. Knowing the levels of ascorbic acid in a food product can help determine the antioxidant properties of the sample[117-120]. A less expensive and reliable way to measure ascorbic acid is with an ascorbate oxidase biosensor [117]

1.5.4 Lactate Oxidase

Lactate oxidase (LOx) from *Aerococcus viridans* is a tetramer with a molecular weight of 162 kDa.[121] LOx catalyzes the oxidation of lactate to pyruvate and hydrogen peroxide in the presence of a flavin mononucleotide (FMN) co-factor. LOx is a member of the α-hydroxy-acid oxidase flavoenzyme[122]. It is used for assays to measure lactate levels for medical diagnostic [123] and in the food industry [124-127] Continuous measurement of lactate using biosensors is limited by enzyme instability.[128,129]

1.5.5 Glucose Oxidase

Glucose oxidase (GOx) from *Aspergillus niger* is a dimer with a molecular weight of 160 kDa. The glycoprotein consists of two flavin adenine dinucleotide (FAD) molecules in each dimer. GOx catalyzes the oxidation β-D-Glucose into the formation of gluconic acid. This process produces hydrogen peroxide by using oxygen as an electron acceptor.[92] GOx has several food industry applications including the removal of glucose from dried eggs, removal of oxygen, improvement of color, texture, shelf life, and flavor.[130,131] Glucose oxidase is used in the development of biosensors but is limited by the stability of the enzyme.[132]

6. Gap in Knowledge

Numerous studies have used HHP to improve enzyme stability. It is still unknown by which specific mechanisms cause HHP to increase thermal stability. A common idea is that the effects of temperature and HHP are antagonistic to each other. Whereas increasing pressure increases order, thereby decreasing entropy, while increasing temperature decreases order, thereby increasing entropy.[3] Recent studies suggest that the size and location of an enzyme's

internal cavities affect how that protein would behave under HHP.[28,133,134] Currently, no mathematical model exists that can predict how HHP will affect a given enzyme based on the molecular structure. Empirical models must be constructed for each enzyme to observe how they are affected by HHP.

In our laboratory, our research aims to characterize the effects of HHP on other oxidase enzymes with potential use in the food industry and that produce hydrogen peroxide allowing them to function in biosensors. The stabilizing effect of HHP on oxidases including glucose oxidase,[86,87] alcohol oxidase,[89] xanthine oxidase,[90] pyruvate oxidase,[91] pectinase,[135] and lipase[3] has been studied. These enzymes experience a stabilizing effect under HHP. Additionally, the effect of HHP was able to be combined with other stabilization methods, such as hydrophobic modification of amino and carboxyl enzyme residues and immobilization for increased biosensor thermal stability. While HHP was able to increase enzyme stability, this stabilization does not persist after depressurization. In order to create a cost-effective and stable biosensor, techniques that maintain stabilized enzyme conformation need to be developed. Therefore, there is a need to study additional enzymes that meet our research criteria. We have identified AsOx, LOx, ChOx, and ClOx as enzymes that meet our criteria, and to the best of our knowledge, the effect of HHP on their stability has not been previously studied.

While our lab has already explored the impact of HHP on glucose oxidase[86,87] the exact mechanism causing HHP to stabilize the enzyme is still unknown. Tryptophan fluorescence can be a useful tool to help better understand protein unfolding.[136,137] To the best of our knowledge, differential scanning fluorometry has not been used to measure the effect of HHP on glucose oxidase.

7. Hypothesis

Hypothesis: HHP will stabilize glucose oxidase, ascorbate oxidase, lactate oxidase, cholesterol oxidase, and choline oxidase against thermal denaturation. This will manifest in an increase of the melting point (T_m) and decrease the rate of thermal inactivation.

Sub-hypothesis 1.1: The optimal pressure determined by the kinetics of inactivation will be between 100 and 300 MPa for each of the enzymes tested.

Sub-hypothesis 1.2: The optimal pressure determined by thermal transition point will be between 100 and 300 MPa for each of the enzymes tested.

8. Specific Objectives

Objective: To determine the optimal pressure of glucose oxidase, ascorbic oxidase, lactate oxidase, cholesterol oxidase, and choline oxidase

- Study 1.1: To determine the optimal pressure of ascorbic oxidase, lactate oxidase, cholesterol oxidase, and choline oxidase via kinetics of thermal inactivation
- Study 1.2: To determine optimal pressure for the stability of glucose oxidase using intrinsic fluorescence

CHAPTER 2

EFFECTS OF HIGH HYDROSTATIC PRESSURE ON THE THERMAL STABILITY OF LACTASE OXIDASE, ASCORBATE OXIDASE, CHOLESTEROL OXIDASE, AND CHOLINE OXIDASE [1]

[1] M.D. To be submitted to a peer-reviewed journal.

Abstract

Enzymes are important catalysts used across numerous industries, including the cosmetic, pharmaceutical, agricultural, and food industries. However, several enzymes lack the stability and the desired recyclability for commercial use. Enzyme biosensors, while a commercial application for enzymes, have operational life and stability limitations that limit their usability. High hydrostatic pressure (HHP), currently used to kill pathogenic microorganisms and to inactivate undesirable enzymes, has been shown to stabilize and increase the activity of some enzymes. This study examines the inactivation kinetics of lactase oxidase, ascorbate oxidase, cholesterol oxidase, and choline oxidase after a 5-min heat treatment that partially inactivated the enzymes at HHP. Pressure reduced the thermal inactivation of lactate oxidase by up to 2.7-fold at 66 °C and 50 MPa, ascorbate oxidase by up to 1.9-fold at 53 °C and 50 MPa, and cholesterol oxidase by up to 16.6-fold at 500 MPa at 67.5 °C compared to atmospheric pressure. Pressure increased the rate of thermal inactivation of choline oxidase by 3.5-fold at 45 °C at 100 MPa compared to atmospheric pressure. Apparent $\Delta V^{\ddagger}$ was positive, favoring partial enzyme stabilization and enzyme folding at 0.1 MPa – 50 MPa for lactate oxidase and ascorbate oxidase, 200 MPa – 400 MPa for choline oxidase, and 0 MPa – 600 MPa for cholesterol oxidase. The apparent $\Delta V^{\ddagger}$ was negative, favoring partial enzyme inactivation and unfolding of the enzyme at pressures 100 MPa - 300 MPa and 100 MPa - 400 MPa for lactate oxidase and ascorbate oxidase, 0.1 MPa – 100 MPa for choline oxidase, and was not observed for cholesterol oxidase. The optimal pressure for lactate oxidase and ascorbate oxidase was between 50 MPa and 100 MPa. Pressure stabilized up to at least 600 MPa for cholesterol oxidase while being a denaturant for choline oxidase.

1. Introduction

In 2021, the global enzyme market was valued at $11.47 billion. As more industries increase the use of enzymes, the enzyme market is predicted to grow at a compound annual growth rate of 6.5%, up to 2030.[138] There are applications for enzymes across an extensive range of industries, not limited to the food,[82] pharmaceutical,[139] cosmetic,[140] agricultural,[141] and chemical industries.[83] The use of enzymes in the food industry has been reviewed thoroughly by Raveendran et al.,[82] including the use in baking,[142,143] alcohol fermentation,[144,145] dairy,[146,147] fruit juice processing,[148,149] and biosensors.[98,102,141,150] Enzymes are useful as they are highly selective and function as biocatalysts. Offering advantages over alternative chemical catalysts, including reducing toxic byproducts, lower cost, higher yield, and improved process control.[82] However, industrial use is limited by the low stability and activity of enzymes in their native state. Enzymes denature and are affected by many environmental factors, including high temperatures, changes in pH, and the presence of denaturants.[9,102,140] Increasing enzyme stability and activity would increase industrial usage as reusability would increase and the biocatalyst cost would decrease. Several methods have been shown to increase enzymatic stability, including immobilization,[151] hydrophobic modification,[152] and high hydrostatic pressure (HHP).[9]

HHP is a nonthermal food preservation process first developed for microbial and enzyme inactivation. Having minimal effects on the nutritional value, taste, appearance, and texture of the treated food[153]. Previous studies have shown certain food enzymes such as lipoxygenase and polyphenol oxidase inactivated at 300 MPa[154,155] and 200 MPa[156] respectively. Despite its inactivating effects, HHP below 400 MPa stabilizes some enzymes against thermal inactivation .The stabilization mechanism is not fully understood.[9,35] Stabilization has been observed in polyphenol oxidase,[157] glucose oxidase,[86] alcohol oxidase,[89] xanthine oxidase,[90] pectin

methylesterase,[157,158] naringinase,[159] and pectinase[135]. The protective effect of HHP allows the enzymes to remain active at higher temperatures, which would have inactivated them at atmospheric pressure. The activity at higher temperatures is also greater. However, the stabilizing effects are only active while pressurized and lost upon depressurization.[88,160] As demand for high-pressure processing equipment grows, the cost of high-pressure processing has decreased, allowing wider industrial implementation, especially within the food industry.[161-164]

Biosensors represent an opportunity for improving clinical diagnostics[165], food safety and processing, [150,166-168] as well as environmental and agricultural monitoring.[141,167] but industrial use is limited due to the lack of stability of enzymes. Oxidase enzymes can be used to fabricate electrochemical enzyme biosensors in the food industry. These amperometric biosensors work on the principle that oxidase catalyzes the substrate with oxygen producing a product plus hydrogen peroxide (eq. 1). The hydrogen peroxide oxidized at the anode correlates to the amount of substrate present.

$$Substate + O_2 \xrightarrow[Catalysis]{Enzyme} Product + H_2O_2$$

The stabilizing effect of HHP on oxidases including glucose oxidase,[86,87] alcohol oxidase,[89] xanthine oxidase,[90] pyruvate oxidase,[91] pectinase,[135] and lipase[3] has been studied. Furthermore, the effect of HHP has been combined with other stabilizing strategies, such as hydrophobic modification of amino and carboxyl enzyme residues and immobilization for increased biosensor thermal stability. Although enzyme stability increased under HHP, stability does not persist after depressurization. To make a more stable and cost-effective biosensor, techniques that retain the stabilized enzyme conformation are still lacking. Furthermore, there is no clear association between the extent of HHP-induced stabilization and structural features of enzymes. Therefore, there is a need to study a larger number of enzymes to elucidate such

correlations. This research aims to characterize the effects of HHP on other oxidase enzymes that produce hydrogen peroxide and have potential use in the food industry, allowing them to function in biosensors. We have identified choline oxidase, lactate oxidase, ascorbate oxidase and cholesterol oxidase enzymes that meet these criteria. Developing strategies for enzyme stabilization and implementing fabrication enhancements to extend reusability, reduce cost, and increase industrial application across the food and agricultural industries.

Choline oxidase (ChOx) from *Alcaligenes sp.* is a dimer with a molecular weight of 120 kDa.[103] ChOx catalyzes the four-electron oxidation of choline to the intermediate betaine-aldehyde to glycine-betaine via a two-step reaction.[104-106] Choline oxidase can be used in a biosensor to determine choline in powdered milk meant for children[107] and is a potential biomarker for early diagnosis of multiple cancers and neurological disorders, but enzyme stability holds ChOx biosensors back from commercialization.[108]

Lactate oxidase (LOx) from *Aerococcus viridans* is tetramer with a molecular weight of 162 kDa.[121] LOx catalyzes the oxidation of lactate to pyruvate and hydrogen peroxide in the presence of a flavin mononucleotide (FMN) co-factor. LOx is a member of the α-hydroxy-acid oxidase flavoenzyme[122]. It is used for assays to measure lactate levels for medical diagnostics[123] and in the food industry.[124-127] Continuous measurement of lactate using biosensors is limited by enzyme instability.[128,129]

Cholesterol oxidase (ClOx) from *Streptomyces* is a monomer[109] with a molecular weight of 62 kDa.[110] ClOx catalyzes the first step of cholesterol catabolism in which cholesterol is oxidized to cholest-5-en-3-one requiring FAD, which then isomerizes to cholest-4-en-3-one with the release of hydrogen peroxide. ClOx is used in the medical industry to monitor human cholesterol levels, which can help reduce the risk of cardiovascular diseases.[111] There are also

applications to be used in the food industry to measure the total cholesterol in foods like eggs, meat, and milk samples.[112-114]

Ascorbate oxidase (AsOx) from *Cucurbita sp* is a dimer with a molecular weight of 140 kDa.[115] AsOx catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. AsOx catalyzes a four-electron reduction of dioxygen to water accompanied by a one-electron of the reducing organic substrate**[116].** AsOx is used in the food and pharmaceutical industries to determine ascorbic acid (vitamin C) concentration. Ascorbic acid is used heavily in the food industry as an antioxidant. Knowing the levels of ascorbic acid in a food product can help determine the antioxidant properties of the sample[117-120]. A less expensive and reliable way to measure ascorbic acid is with an ascorbate oxidase biosensor.[117]

Despite their use in biosensors and colorimetric assays, ChOx,[108] LOx,[127] AsOx,[169] and ClOx[170] enzyme stability presents a limiting factor. Various procedures have studied ChOx, LOx, AsOx, and ClOx stabilization, including protein engineering[171-173] and immobilization.[174-177] To the best of our knowledge, the effect of HHP on ChOx, LOx, AsOx, and ClOx stability against thermal inactivation has yet to be explored. We hypothesized that HHP would improve the stability of ChOx, LOx, AsOx, and ClOx against thermal inactivation. This study aims to determine the optimal pressure that maximizes the stabilization effect on ChOx, LOx, ClOx, and AsOx. This research will provide additional information to understand HHP's effect on the thermal stability of oxidoreductase enzymes overall. This information could provide key information to allow for the development of more stable biosensors.

2. Materials and Methods:

2.1 Materials and Equipment

Choline oxidase from *Alcaligenes sp.*, lactate oxidase from *Aerococcus viridans*, ascorbate oxidase from *Cucurbita*, Peroxidase from horseradish, Trizma base, choline chloride, phenol, 3,3-dimethylglutaric acid, flavinn adenine dinucleotide, bovine albumin, o-dianisidine dihydrochloride, Triton^{TM} X-100, cholesterol and 4-aminoantipyrine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholesterol oxidase was purchased from MP Biomedicals (Eschwege, Germany). Ethylenediaminetetraacetic acid, lactic acid, N, N-dimethylaniline, potassium phosphate, sodium phosphate, hydrochloric acid, L-ascorbic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

The HHP equipment used was originally reported by Halalipour.[86] The HHP system consists of a high-pressure micro *pump* (model MP5), an 8.5-mL high-pressure reactor (model U111), and a pump controller (MP5 micropump control unit) from Unipress Equipment (Warsaw, Poland). Water baths, Isotemp 6200 H11 (20 °C – 80 °C) and Isotemp 6200 R28 (10 °C) from Fischer Scientific (Pittsburg, PA, USA) fed water into the jacket of the high-pressure reactor to control the temperature of the sample chamber. The high-pressure reactor was filled with silicone oil to act as a pressurization fluid. The water flow from the water baths was controlled by a pair of Sirai Z110A solenoid pinch values (Busseri, Italy). A type K thermocouple was inserted through the bottom of the reactor to monitor the temperature within the reactor. A computer program written in LabVIEW was used to collect process time, temperature, pressure, data as well as to actuate the solenoid valves. The high-pressure controller, the thermocouple, and the solenoid valves were interfaced through a data acquisition system that includes an NI cDAQ 9174 chassis with NI 9211, NI 9215, NI 9263, and NI 9481

modules from National Instruments (Austin, TX, USA). The HHP system is depicted in Figure 2.1.

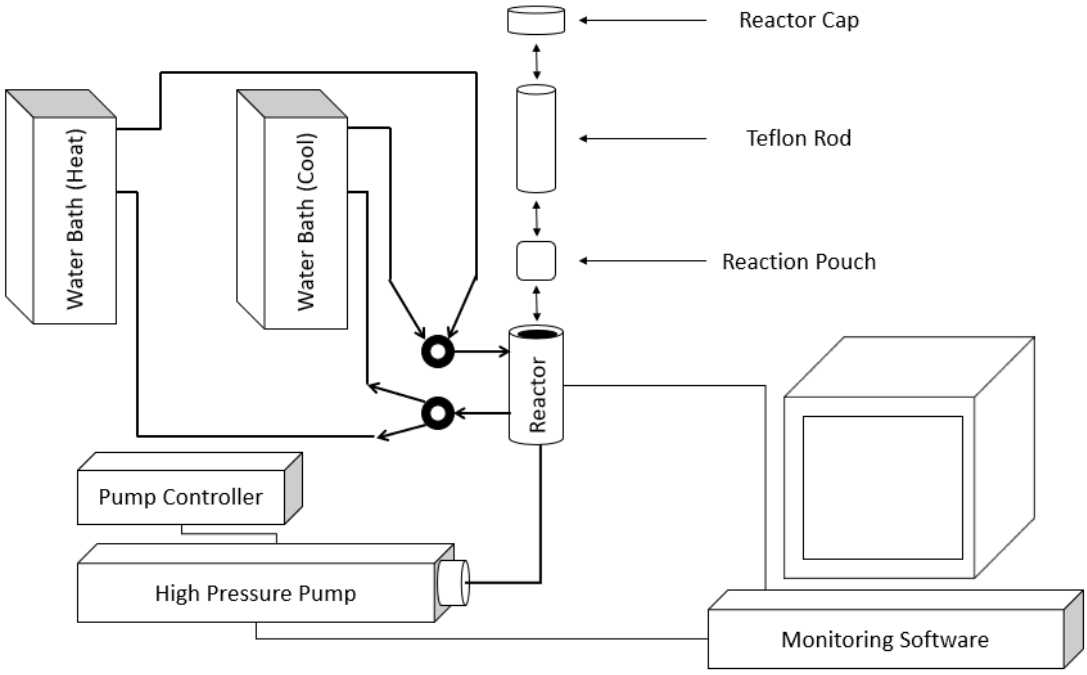

Figure 2.1 Schematic representation of the high hydrostatic pressure system

2.2 Methods

2.2.1 Enzyme Activity Assays

Enzymatic assays were adapted from Sigma protocols and references therein. Peroxidase (POD)-coupled reactions in which hydrogen peroxide reacts with a dye were used for all the enzymes that do not consume or produce a chromogenic reactant or product. Enzyme activity was determined as the rate of change in absorbance *vs.* time. Assays were adapted for the microplate reader by reducing 10-fold the volume of the reaction mixture and were carried out at 25 °C. Three wells, one blank and two treated enzyme analytical replicates, were recorded for each assay. Analytical replicates of the slope of absorbance vs. time were averaged for the

recorded value. Spectrophotometry was done using a BioTek Synergy™ HTX Multi-Mode Microplate Reader (Winooski, VT, USA). Data was collected using BioTek's Gen5 Data Analysis Software.

ChOx activity was measured as the production rate of quinonimine dye at 500 nm as described elsewhere.[178,179] In brief, first 40 µL of a treated 0.5 U/mL ChOx in 10 mM Tris HCl with 2.0 mM EDTA and 134 mM potassium chloride at pH 8.0, was pipetted into a 96-well plate. Using the auto-injector coupled to the microplate reader, 160 µL of reaction cocktail, containing 97 volumes of 2.1% (w/v) choline chloride in 100 mM Tris HCl buffer at pH 8.0; 1 volume of 1% (w/v) 4-Aminoantipyrine in DI water; 2 volumes of 1% (w/v) phenol in DI water; and 500 units of horseradish POD was added to the plate. The plate was shaken for 30 s at 25° C by the plate reader. Absorbance was measured at 500 nm for 5 min recording absorbance *vs.* time. Final concentrations were 96 mM Tris, 2.0% (w/v) choline, 0.01% (w/v) 4-aminoantipyrite, 0.02 % (w/v) phenol, 15 units POD, 0.03 mM ethylenediaminetetraacetic acid, 2 mM potassium chloride and 0.025-unit choline oxidase. All chemical solutions were stored on ice or at 4 °C when not in use.

LOx activity was measured as the production rate of quinonimine dye at 565 nm as described elsewhere.[122] In brief, 20 µL of treated LOx, 0.2 U/mL LOx, 10 mM potassium phosphate buffer with 0.010 mM FAD at pH 7.0 was pipetted into a 96 well plate. Using the auto-injector coupled to the microplate reader, 180 µL of reaction cocktail, containing 2 volumes of 200 mM 3,3-dimethylglutaric acid-NaOH buffer (DMGA) at pH 6.5; 1 volume of 50 un/mL POD in DI water; 1 volume of 15 mM 4-Aminoantipyrine in DI water; 1 volume of 500 mM L(+)lactic acid at pH 6.5; and 3 volumes of DI water, was added to the plate. The plate was shaken for 30 s at 25 °C by the plate reader. Absorbance was monitored at 565 nm for 5 min

recording absorbance vs. time. The final concentration was 39 mM 3,3dimethylgluaric acid, 5 units PODx, 1.5 mM 4-aminoantipyrine, 49 mM L(+) lactic acid, 0.04% (v/v) N,N dimethylamine, 0.20 mM potassium phosphate, 0.20 µm FAD and 0.004 unit lactate oxidase. All chemical solutions were stored on ice or at 4 °C when not in use.

AsOx activity was measured as the production rate of L-dehydroascorbic acid at 245 nm and described elsewhere.[180] In brief, the change in absorbance was recorded immediately after 40 µL of treated AsOx, 2.4 U/mL in 4mM sodium phosphate buffer with 0.05% (w/v) bovine serum albumin at pH 5.6 was pipetted into a 96-well plate. Using the auto-injector coupled to the microplate reader, 260 µL of the ascorbic acid solution, 0.5 mM L-ascorbic acid in 100 mM potassium phosphate with 4 mM sodium phosphate dibasic and 0.5 mM ethylenediaminetetraacetic at pH 5.6 was injected into the wells. The plate was shaken for 30 s at 25 °C by the plate reader. Absorbance was monitored at 245 nm for 5 min recording absorbance vs. time. The final concentration was 91 mM potassium phosphate, 0.45 mM L-ascorbic acid, 0.5 mM ethylenediaminetetraacetic acid, 4 mM sodium phosphate, 0.005% (w/v) bovine serum albumin, and 0.024 unit of ascorbate oxidase. All chemical solutions were stored at 4 °C when not in use. The ascorbic acid solution was made fresh daily and standardized using 200 mM HCl, $A_{245} = 1.25 \pm 0.05$.

ClOx activity was measured as the oxidation rate of o-Dianisidine at 500 nm.[181] In brief 10 µL of 100 U/mL POD in DI water and 10 µL of 0.5 (w/v) cholesterol with 10% (w/v) Triton™ -X-100 Solution in DI water were combined by pipetting them into a 96-well plate. Using auto-injector 1 coupled to the microplate reader 240 µL of reaction cocktail, 440 µM of o-dianisidine in 50 mM potassium phosphate buffer at pH 7.5, was injected into the plate. Plate was shaken for 60 s at 25 °C by the plate reader. The change in absorbance was recorded

immediately after 40 µL of treated enzyme, 0.2 un/mL cholesterol oxidase 50 mM potassium phosphate buffer at pH 7.5, was pipetted into a 96-well plate. Absorbance was monitored at 500 nm for 5 min recording absorbance *vs.* time. The final concentration was 46 mM potassium phosphate, 0.009% o-dianisidine, 0.017% (w/v) cholesterol, 0.33 (v/v) Triton X-100, 10 un peroxidase, and 0.02 un cholesterol oxidase. Chemical solutions were stored on ice or at 4 °C when not in use. The reaction cocktail was made fresh daily.

2.2.2 High Hydrostatic Pressures Effect on Thermal Inactivation of Enzymes

Aliquot bags made of low-density polyethylene were cut into 2 cm x 2 cm squares and heat-sealed together on three sides to form a pouch. For each treatment, pouches were filled with either 160 µL, 80 µL, 160 µL, or 160 µL of AsOx, LOx, ClOx, and ChOx, respectively. Pouches were heat-sealed and either placed on ice or immediately into the HHP reactor, kept at 10 °C. The reactor was topped off with silicon oil before being closed.

At the beginning of the treatment, the high-pressure cell was cooled to 10 °C at atmospheric pressure. Then the cell was pressurized. When the pressure in the cell reached the pressure setpoint, the temperature was increased by switching the flow of water into the cell from the cold-water bath held at 10 °C to the hot water bath maintained at the desired treatment temperature. Treatment time of either 0 min or 5 min would begin when cell temperature reached 95% of the treatment temperature. After the expected incubation time, the cell was first cooled to 15 °C by switching the flow of water into the cell from the hot water bath to the cold one. Immediately after reaching 15 °C, the cell was depressurized and opened, and the sample was retrieved. Then the enzyme activity assay was conducted. Figure 2.1 shows the pressure and temperature profiles of the treatment of cholesterol oxidase at 200 MPa for an incubation time of 0 min (open symbols) and 5 min filled symbols.

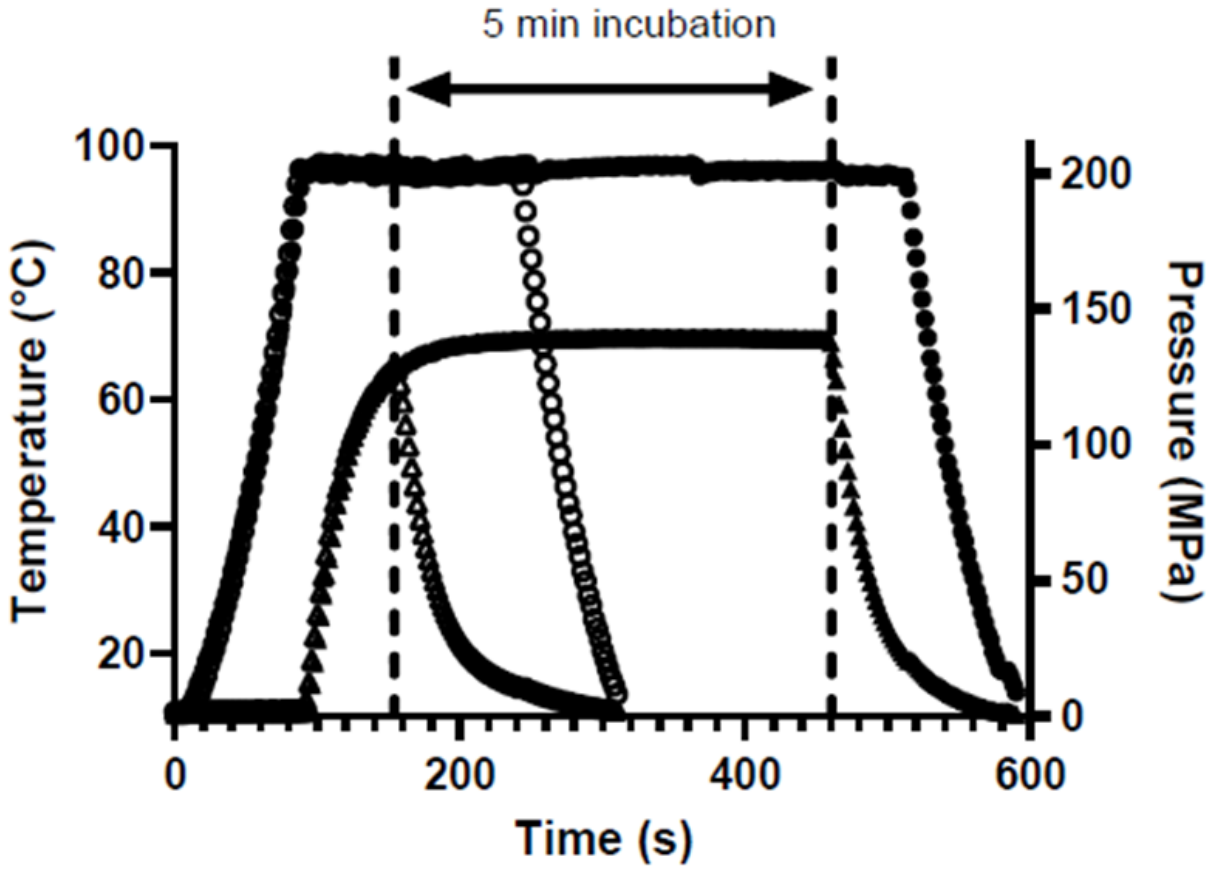

Figure 2.2. Heating and pressurization diagram for cholesterol oxidase temperature (△) and pressure (○) of 0 min incubation time at 67.5 ℃ and 200 MPa; and temperature (▲) and time (●) for 5 min incubation time at 67.5 ℃ and 200 MPa.

Previous research in our group with GOx,[86] XOx,[90] and AOx,[89] concluded that the optimal pressure did not vary significantly in the range of temperatures studied. Therefore, experiments were conducted at a single temperature for this study. For each of the enzymes of this study, preliminary experiments were conducted to determine the temperature that produces

an ~80% thermal inactivation after 5 min. The percent residual activity was calculated as the ratio of activity after a 5-min treatment over the activity of incubation time of 0 min:

$$Acty_{res} = \left(\frac{Acty}{Acty_0}\right) * 100$$

Treatment temperatures for AsOx, LOx, ClOx, and ChOx were 53 °C, 66 °C, 67.5 °C, and 45 °C, respectively.

Experiments for each enzyme were initially conducted in a completely randomized design with each pressure being conducted in triplicate. The initially tested pressures for each enzyme were 0.1 MPa, 100 MPa, 200 MPa, 300 MPa, and 400 MPa. Additional pressures were added to examine levels of interest within or outside the initial range of pressures. These pressures were 50 MPa and 150 MPa for AsOx and LOx and 500 MPa and 600 MPa for ClOx. Statistical analysis was completed using GraphPad Prism (San Diego, CA) using analysis of variance (ANOVA) and Tukey's pairwise comparison at a confidence level corresponding to α =0.05.

2.2.4 Rate Constant of Inactivation and Activation Volume

A coarse estimate of the pseudo first-order rate constant of inactivation was done using the activity at treatment time 0 min and the residual activity after only one treatment time. This is not a robust approach but was used to obtain order of magnitude estimates of the activation volume or inactivation. Determination of additional treatment times was out of the scope of this study. Therefore, the linearized version of the Eyring Equation was used to determine the activation volume.

$$\ln\left(k_{inact}\right) = \left(-\frac{\Delta V^{\ddagger}}{RT} \times P\right) + \ln\left(k_{inact,P_0}\right)$$

Where $\Delta V^{\ddagger}$ is the activation volume, P is the pressure, and k_{inact,P_0} is the rate of enzyme inaction at the reference pressure P_0.[182] Error estimates for k_{inact} and activation volume were determined by using the standard error of the slopes of the linear regression.

3. Results and Discussion

3.1. Effect of HHP on the Stability of Enzymes

To the best of our knowledge, no other studies have examined the effect of pressure on the thermal inactivation of these enzymes. All enzymes were subjected to a 5-min treatment under the selected pressures. The residual activities of the treated enzymes were compared to those treated for 0 min at the same pressure. Figure 2 shows the effect of pressure on the residual activity for (A) LOx, (B) AsOx, (C) ChOx, and (D) ClOx after thermal treatments. The residual activities of all the enzymes tested were significantly ($p<0.05$) affected by pressure as determined by a Tukey's test ($\alpha = 0.05$) between ambient pressure and pressure treatments.

Thermal stability results at ambient pressure were consistent with other studies reported to the Brenda Enzyme database. AsOx from *Cucurbita sp.* was reported to be thermally stable at 55 °C at pH 6.5 for 30 min, with an optimal pH of 7.0 at 30 °C for 60 min. When the pH was lowered, a reduction in stability was observed.[183] ClOx from *Streptomyces sp.* was reported to be thermally stable between 30 – 40 °C at pH 7.0 for 120 min,[184] less than a 50% residual activity after 15 min at 55 °C,[185] and an optimal pH of 9 was stable up to 60 °C for 10 min.[186] LOx from *Aerococcus viridans* was reported to be thermally stable at 45 °C for 10 min with LOx experienced 50% reduction in residual activity at 55 °C after 10 min.[187] ChOx from *Alcaligenes sp.* was reported to be thermally stable below 40 °C at pH 7.5 for 10 min.[188,189]

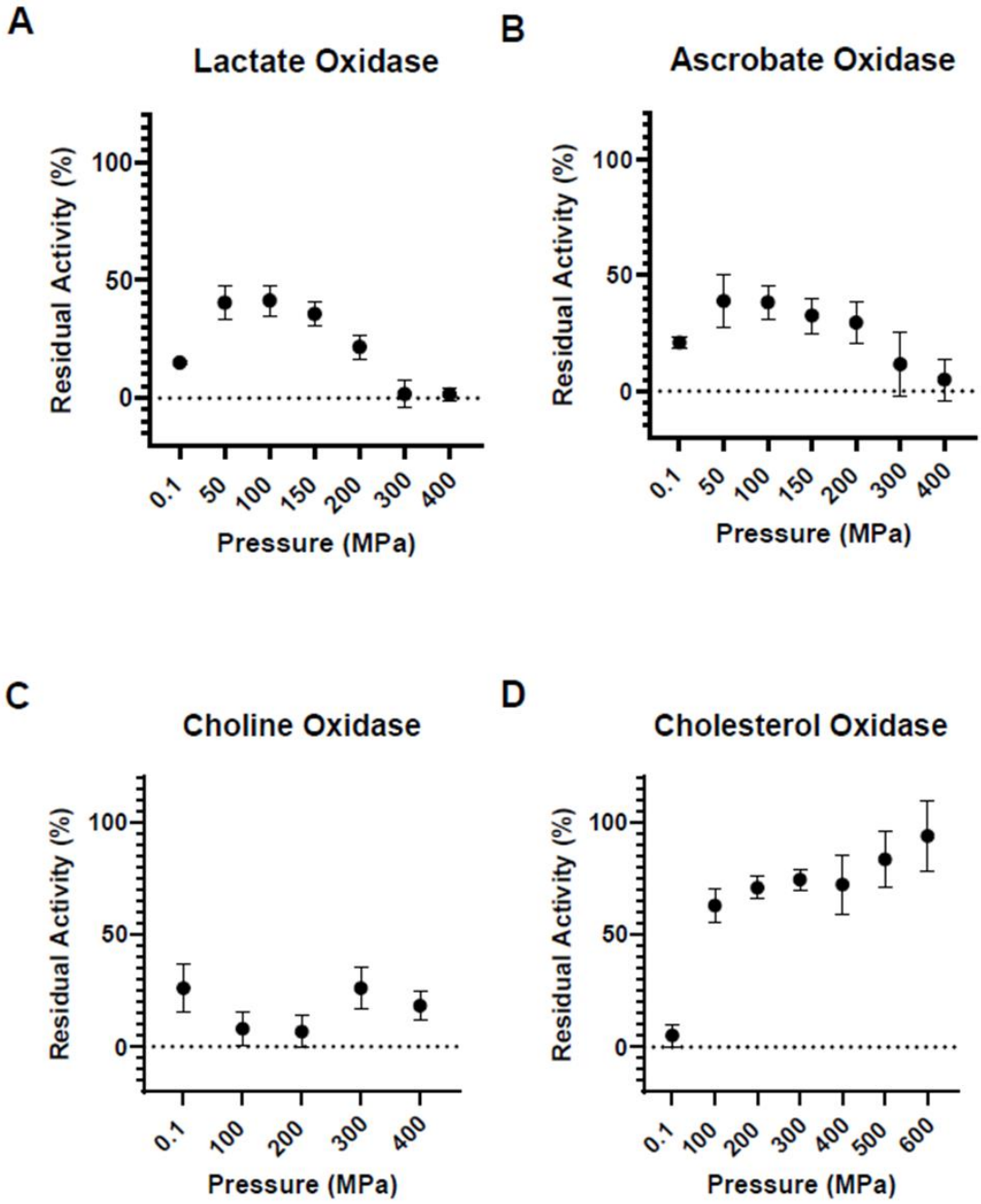

Figure 2.3 Effect of high hydrostatic pressure on residual activity (A) lactate oxidase at 66 °C, (B) ascorbate oxidase at 53 °C, (C) choline oxidase at 45 °C, (D) cholesterol oxidase at 67.5 °C after 5 min incubation time. Error bars represent a 95% confidence interval determined by Tukey's test.

Lactate oxidase was treated at 66 °C for 5 min from 0.1 MPa to 400 MPa (Figure 2.3A). At atmospheric pressure, after 5-min treatment time LOx's residual activity was ~15%. As pressure increased, LOx activity increased and reached its maximum of ~ 40% between 50 MPa, 100 MPa, and 150 MPa, but experiments lacked sufficient resolution to determine a single pressure as the maximum, only providing a pressure range. Residual activity at 50 MPa, 100 MPa, and 150 MPa was significantly higher ($p<0.05$) than at 0.1 MPa. There was no significant difference ($p<0.05$) between 50 MPa, 100 MPa, and 150 MPa. LOx's thermal inactivation decreased significantly ($p<0.05$) by a factor of 2.5, when pressurized between 50 MPa, 100 MPa, and 150 MPa. Residual activity was at ~2% between pressures 300 and 400 MPa. This could be caused by a complete inactivation of the enzyme past 300 MPa due to a pressure-induced irreversible conformational change.

Ascorbate oxidase was treated at 53 °C for 5 min from 0.1 MPa to 400 MPa (Figure 2.3B). After a 5-min treatment time, AOx residual activity at atmospheric pressure was 21%. Ascorbate Oxidase's relative residual activity increased until it reached its maximum between 50 MPa, 100 MPa, and 150 MPa with residual activity of 39%, but, like for LOx, there were no significant differences in the residual activity between 50 -150 MPa. However, residual activity at these pressures was significantly higher ($p<0.05$) than atmospheric pressure. At 50 MPa, the thermal inactivation of the ascorbate oxidase decreased by a factor of 0.86 compared to 0.1 MPa. At 300 MPa, significant pressure inactivation was observed. Thermal inactivation increased by a factor of 1.7 compared to 50 MPa.

Choline oxidase was treated at 45 °C for 5 min from 0.1 MPa to 400 MPa (Figure 2.3C). After 5-min treatment time residual activity was 26%. Choline oxidase's relative residual activity did not significantly increase with pressure; the opposite occurred. It decreased until it reached a

minimum residual activity of 8% at 100 MPa. Residual activity at 100 MPa pressure was significantly lower ($p<0.05$) than ambient pressure. The thermal inactivation of the choline oxidase enzyme decreased by a factor of 3.3, showing significant ($p<0.05$) pressure-induced inactivation at 100 MPa and 200 MPa. The thermal inactivation stayed at that level until 300 MPa, when it returned to not being significantly ($p<0.05$) different from the atmospheric pressure. To the best of our knowledge, no previous studies have shown a similar increase in inactivation followed by a stabilization as pressure increased. We hypothesize that HHP is causing ChOx not to denature but rather to aggregate.

Cholesterol oxidase was treated at 67.5 °C from atmospheric pressure 0.1 – 600 MPa in increments of 100 MPa for 5 min. Figure 2.3D shows that after a 5-min treatment at 0.1 MPa, the residual activity was 5%. Cholesterol oxidase's relative residual activity increased from 0.1 MPa to 600 MPa without experiencing a decrease, reaching a residual activity of 94%. Residual activity at 100 MPa was significantly ($p<0.05$) higher than atmospheric pressure. Compared to a similar incubation at atmospheric pressure, at 600 MPa, the thermal inactivation of cholesterol oxidase was reduced 16.6 times. Thermal inactivation did not decrease significantly from 100 MPa – 600 MPa.

Several studies have described the impact of pressure on the inactivation of other enzymes. Previous studies have shown that HHP stabilize enzymes at pressures typically below 300 MPa while destabilizing them above that pressures.[9,190] Pressure-induced stabilization of lactate oxidase and ascorbate oxidase were observed to be similar to glucose oxidase,[86-88] xanthine oxidase,[90] α-chymotrypsin,[35] and inulin fructotransferase.[160] These enzymes experienced an increase in stability compared to atmospheric pressure as pressure increased up until a critical value, and after that point, a pressure-induced denaturation occurred. Glucose

oxidase thermal stability increased under HHP up to 240 MPa, and pressure became a denaturant between 240 MPa and 360 MPa, indicating an optimal pressure for stability ~240 MPa.[87] The thermal stability of xanthine oxidase increase by a factor of 9.5 at 300 MPa compared to atmospheric pressure at the same temperature, 70 °C.[90] Increased thermal stability of α-chymotrypsin was observed. At 50 °C and treatment times up to 40 min, the residual activity measured as inactivation half-life in the paper increased from less than 5 min at atmospheric pressure to 15 min at 60 MPa and 30 min at 120 MPa maintaining thermal stability up to 180 MPa.[35] The thermostability of inulin fructotransferase from *Arthrobacter aurescens* was observed from 60 – 80 °C at 200 MPa compared to atmospheric pressure. At 200 MPa thermal stability increased by 13.2% at 60 °C up to 220% at 80 °C and a treatment time of 15 min compared to atmospheric pressure. At 60 °C, as pressure increased to 300 MPa, residual activity returned to similar levels as those observed at atmospheric pressure. Thermal inactivation increased by 78.8% at a pressure of 400 MPa.[160]

The pressure-induced stabilization of cholesterol oxidase was shown to be like that of immobilized lipase in hexane[3] and A. *aculeatus* pectin methylesterase.[191] Where HHP had a stabilizing effect at 600 MPa. After a 4-h incubation at 80 °C, immobilized lipase saw a significant reduction in thermal inactivation from 10 – 300 MPa. The decrease in thermal inactivation peak came when comparing the residual activity from ambient pressure to 400 MPa, with a total reduction of 2.5 fold. As the pressure increased, thermal inactivation remained similar until a final tested pressure of 700 MPa.[3] A. *aculeatus* pectin methylesterase (PME) experiences a similar reduction in thermal inactivation. A. *aculeatus* PME treated for at 55 °C and atmospheric pressure was compared to pressures from 100 – 700 MPa at 55 °C. After 10 min, the atmospheric treatment residual activity was reduced by 90%, while after over 30 min,

pressures 100 MPa, 400 MPa, and 700 MPa showed residual activities above 75, 90, and 90%, respectively.[191]

Our residual activity findings allowed determining the effect of HHP on the thermal inactivation of the selected enzymes. However, residual activity does not provide any information about the structural changes that enzymes undergo during their thermal inactivation at atmospheric pressure or at HHP. Further testing is required to better understand the structural changes undergoing lactate oxidase, ascorbate oxidase, choline oxidase, and cholesterol oxidase. Analyses that would complement this research include but are not limited to fluorescence to characterize protein folding, nuclear magnetic resonance spectroscopy to study the changes in conformation, denaturation and internal mobility, circular dichroism spectroscopy to examine the secondary structure and folding properties, Fourier transform spectroscopy to examine the secondary structure and local conformational changes, and SAXS to measure radius of gyration and folding states but were out of scope for this project.

3.2 Activation Volume

The rate constant of inactivation k_{inact} used to calculate $\Delta V^{\ddagger}$ was calculated using the activity of the enzyme after 0 min and 5 min treatments. Thus, results give an order of magnitude approximation. The effect of pressure on k_{inact} was calculated for all four enzymes, LOx at 0.1 – 400 MPa at 66 °C, AsOx at 0.1 - 400 MPa at 53 °C, ChOx at 0.1 – 400 MPa at 45 °C and ChOx at 0.1 – 600 MPa at 67.5 °C. The value of k_{inact} at each pressure are reported in Table 2.1 Apparent $\Delta V^{\ddagger}$ for each enzyme was determined by finding the ranges of pressure in which $\ln(k_{inact})$ changed linearly with respect to pressure slope, on Eyring's plot (Figure 2.3). Activation volumes are reported in Table 2.2. When the activation volume is positive, pressure stabilizes the enzyme, while a negative activation volume inactivates the enzyme.[17] There is a

deviation from Eyring's model as the pressure effect on the enzymes shifts between inactivation and stabilization.[192]

Table 2.1 k_{inact} of lactate oxidase, ascorbate oxidase, choline oxidase, and cholesterol oxidase at pressures 0.1 – 600 MPa

Pressure (MPa)	Enzyme			
	Lactate Oxidase	Ascorbate Oxidase	Choline Oxidase	Cholesterol Oxidase
	k_{inact} (min^{-1}) $\times 10^2$			
.01	17.0 ± 1.8	15.8 ± 2.2	14.8 ± 0.78	19.0 ± 1.7
50	11.9 ± 0.86	12.2 ± 1.5		
100	11.7 ± 1.4	12.3 ± 8.8	18.4 ± 8.9	7.41 ± 4.5
150	12.9 ± 0.88	13.5 ± 0.86		
200	15.9 ± 2.3	14.1 ± 0.67	18.7 ± 0.40	5.84 ± 1.5
300	19.8 ± 2.4	17.7 ± 1.2	14.8 ± 2.8	5.11 ± 3.2
400	20.2 ± 1.3	19.0 ± 3.1	16.4 ± 2.3	5.56 ± 2.7
500				3.31 ± 1.4
600				1.24 ± 5.3

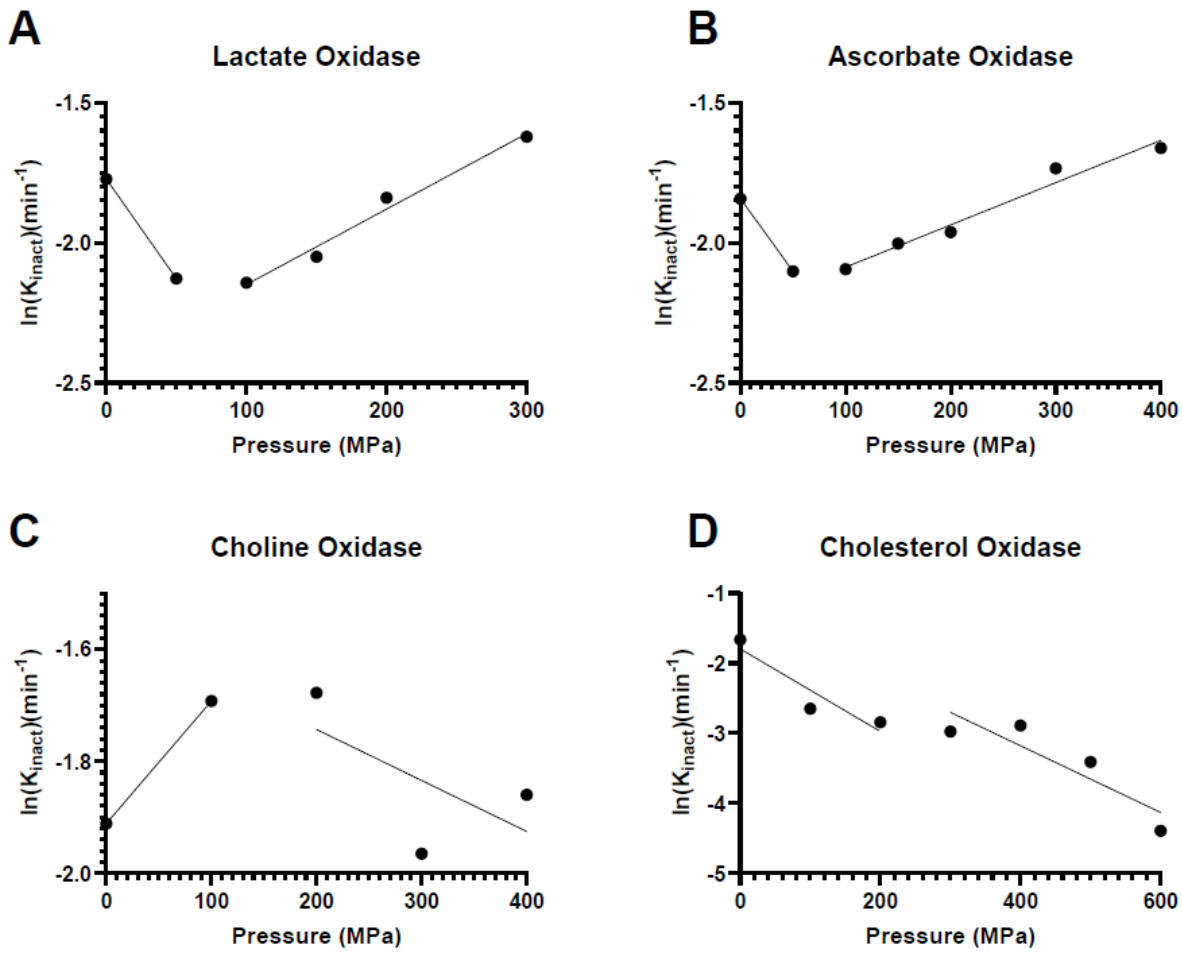

Figure 2.4. Eyring's plot for the rate constant of inactivation (A) lactate oxidase at 66 °C, (B) ascorbate oxidase at 53 °C, (C) choline oxidase at 45 °C, (D) cholesterol oxidase at 67.5 °C after 5 min incubation time.

Table 2.2 $\Delta V^{\ddagger}$ of lactate oxidase, ascorbate oxidase, choline oxidase, and cholesterol oxidase.

	Enzyme			
	Lactate Oxidase	Ascorbate Oxidase	Choline Oxidase	Cholesterol Oxidase
	$\Delta V^{\ddagger}$(cm^3 mol^{-1})			
Stabilization	20.0	14.1		9.90
Inactivation	-7.60	-4.81		

Lactate oxidase and ascorbate oxidase experienced similar pressure effects on the rate constant of inactivation (Figure 2.4A and 2.4B). As pressure increased from 0.1 MPa – 50 MPa, the rate constant of inactivation decreased from 0.17 ± 0.018 min^{-1} – 0.119 ± 0.0086 min^{-1} and 0.158 ± 0.0022 min^{-1} – 0.122 ± 0.015 min^{-1} respectively, which indicated that pressure stabilized both enzymes. The $\Delta V^{\ddagger}$ of stabilization was 20 cm^3 mol^{-1} and 14.1 cm^3 mol^{-1} for LOx and AsOx respectively. The ln(k_{inact}) vs pressure deviated from linearity and leveled off between 50 MPa – 100 MPa. This change reflects a switch from pressure-induced stabilization to pressure-induced inactivation. From 100 MPa – 300 MPa for lactate oxidase and 100 MPa – 400 MPa for ascorbate oxidase ln(k_{inact}) *vs.* pressure became linear but with a positive slope as pressure favored the thermal inactivation. The rate constant of inactivation increased from 0.117 ± 0.014 min^{-1} – 0.198 ± 0.024 min^{-1} and 0.123 ± 0.015 min^{-1} – 0.19 ± 0.031 $min^{-1}$for LOx and AsOx and the $\Delta V^{\ddagger}$ was -7.60 cm^3 mol^{-1} and -4.81 cm^3 mol^{-1} respectively.

For choline oxidase (Figure 2.4C) there was an observed an increase in the rate constant of inactivation from 0.1 MPa – 100 MPa from 0.148 ± 0.0078 min^{-1} - 0.184 ± 0.0089 min^{-1}. With a $\Delta V^{\ddagger}$ of -5.94 cm^3 mol^{-1}. Between 100 MPa – 200 MPa ln(k_{inact}) *vs.* pressure deviated from linearity as pressure switched from inactivating to stabilizing the enzyme from 200 MPa – 400

MPa. The rate constant decreased from 0.187 ± 0.0040 min^{-1} - 0.164 ± 0.023 min^{-1} as pressure appeared to favor stabilization. The $\Delta V^{\ddagger}$ was 2.47 $cm^3\,mol^{-1}$.

Cholesterol oxidase's (Figure 4D) rate constant of inactivation decreased from 0.19 ± 0.017 min^{-1} - 0.124 ± 0.053 min^{-1} from 0.1 – 600 MPa with an overall $\Delta V^{\ddagger}$ of stabilization of 9.90 $cm^3\ mol^{-1}$. Between 0.1 MPa and 600 MPa $\ln(k_{inact})$ *vs.* pressure showed two apparently linear regions between 0.1 MPa and 200 MPa, $\Delta V^{\ddagger}$ of 16.70 $cm^3\ mol^{-1}$, and between 300 MPa and 600 MPa, $\Delta V^{\ddagger}$ of 13.50 $cm^3\ mol^{-1}$. Between 200 MPa and 300 MPa, the effect of pressure leveled off. However, ClOx only experienced stabilization effects from the HHP treatments.

The apparent $\Delta V^{\ddagger}$ of stabilization of LOx, AsOx, and ClOx had a similar order of magnitude to glucose oxidase,[86] alcohol oxidase,[89] and xanthine oxidase[90] and recorded in Table 2.3. There does not appear to be any correlation between their apparent $\Delta V^{\ddagger}$ of stabilization molecular weight or quaternary structure.

Table 2.3. The apparent $\Delta V^{\ddagger}$ of stabilization compared to enzyme structural information, molecular weight and quaternary structure, of glucose oxidase, xanthine oxidase, lactate oxidase, alcohol oxidase, ascorbate oxidase, and cholesterol oxidase

Enzyme	$\Delta V^{\ddagger}$ of Stabilization ($cm^2\ mol^{-1}$)	Molecular Weight (kDa)	Quaternary Structure
Glucose oxidase	57	160	Dimer
Xanthine oxidase	28.9	283	Dimer
Lactate oxidase	20.0	80	Tetramers
Alcohol oxidase	20.0	600	Homo-octamer
Ascorbate oxidase	14.1	140	Dimer
Cholesterol oxidase	9.9	34	Monomer

4. Conclusion

This chapter describes the determination of optimal pressure of ascorbate oxidase, lactate oxidase, cholesterol oxidase, and choline oxidase by using the kinetics of thermal inaction. Pressure's effect on enzyme stability differed for each enzyme. An optimal pressure was determined for LOx and AsOx to be between 50 – 100 MPa, with enzymes experiencing a 1.5- and 1.3-fold increase in stability, respectively. ClOx was stabilized up to at least 600 MPa with no observable max increasing enzyme stability 15.8-fold. While ChOx was partially denatured by pressure with no clear stabilization effect. There does not appear to be a clear association between optimal pressure and enzyme size, quaternary structure, or total enzyme cavity volume.

CHAPTER 3

DESIGN AND CONSTRUCTION OF A PRESSURE SYSTEM TO MEASURE INTRINSIC FLUORESCENCE AND THE STUDY OF THE AGGREGATION OF GLUCOSE OXIDASE AT HIGH HYDROSTATIC PRESSURE [1]

[1] M.D. To be submitted to a peer-reviewed journal.

Abstract

Observing conformational changes of proteins under pressure is critical to help understand the mechanisms of High Hydrostatic Pressure (HHP) stabilization. An HHP system was built to monitor intrinsic fluorescence between atmospheric pressure and 300 MPa. Intrinsic fluorescence was observed at ex 280 nm, em 300 – 400 nm while the temperature was increased 1 °C min^{-1}. Glucose oxidase (GOx) did show a well-defined transition with increasing fluorescence, suggesting protein aggregation at temperatures above 69 °C. The transition temperature T' increased as pressure increased from atmospheric pressure up to 300 MPa. At atmospheric pressure, T' was 69.6 ± 0.5 °C and increased to 76.0 ± 0.2 °C at 100 MPa. This was a ΔT' of 6 °C. A positive shift in ΔT' suggests that pressure prevents protein aggregation.

1. Introduction

Glucose oxidase (GOx) from *Aspergillus Niger*, is a dimeric flavoprotein with a molecular weight of 160 kDa. GOx catalyzes the oxidation β-D-Glucose into the formation of gluconic acid. This process produces hydrogen peroxide by using oxygen as an electron acceptor.[193,194] GOx is used in the food industry with applications including egg powder,[195] seafood storage,[93] baking,[196] gluconic acid production, and beverages.[197] For egg, GOx catalyzed the oxidation of glucose thus limiting Maillard browning during spray drying. Indeed, in the presence of glucose or other reducing sugars, proteins and heat, Maillard reactions lead to the formation of unwanted flavor and color.[198] GOx has also been used to control microbial growth in liquid whole eggs. This inhibition is linked to the bacteriostatic effect of hydrogen peroxide due to the peroxidation of membrane lipids and not the change in pH due to gluconic acid production.[195] This cannot be generalized as studies with shrimp[199] and fish[93] have linked the extension of seafood shelf life to hydrogen peroxide as well as the pH change associated with

gluconic acid production. Adding GOx during bread making allows wheat flour proteins to crosslink, mostly the albumin and globulin fractions, improving the baked goods' functional properties.[196]

Outside of the food industry, GOx applications include use in textiles (bio-bleaching), fuel cells (biofuel cells), chemical (immunoassays and staining procedures), and medical fields (assays and biosensors).[80,94,200] The most commercially practical use for glucose oxidase is measuring blood glucose in a biosensor. However, glucose oxidase biosensors are limited by the stability of the enzyme.[132]

Previous studies have shown that High Hydrostatic Pressure (HHP) increased the stability of enzymes including glucose oxidase,[86,87] alcohol oxidase,[89] xanthine oxidase, pyruvate oxidase,[91] pectinase cocktail,[135] and lipase.[3] However, HHP's stabilizing effect against thermal inactivation only occurs while under pressure. It is difficult to know how exactly pressure stabilizes and inactivates enzymes using the current structural information. The exact mechanism of HHP-induced stabilization and activation is not fully understood.[9]

Protein unfolding and aggregation can be described using a three-state model. Upon unfolding the protein can also undergo aggregation, which can be reversible or irreversible.

$$N \overset{K_1}{\leftrightarrow} U \overset{k_2}{\rightarrow} A$$

Where (N) is the native protein, (U) is the unfolded or partially unfolded protein, (A) represents the aggregated state, and (K_2) is the rate constant of aggregation. As the protein unfolds, hydrophobic residues are exposed, causing aggregation. This causes the protein to become kinetically locked in the aggregated state. The 3-state model can often be simplified to a 2-state irreversible model:[201,202]

$$U \xrightarrow{k_{app}} A$$

Fluorescence is commonly used to characterize the effect of pressure on proteins.[203] Intrinsic fluorescence due to abundant tryptophan and tyrosine residues of some proteins can be used to determine the protein folding state as a function of temperature. One of the main advantages of using fluorescence spectroscopy is that it can observe environmental effects on protein interactions.[204] Typically, intrinsic fluoresce is measured as pressure is increased at constant temperature in a high-pressure cell with sapphire windows.[205,206] Differential scanning fluorimetry (DSF) can be used to characterize a protein's thermal stability and folding state.[207] To our knowledge, no DSF studies have been conducted at HHP. This study aimed to design and construct an HHP system capable of measuring intrinsic fluorescence. The HHP system was then used to determine the impact of HHP during thermal inactivation on the stability of glucose oxidase at high pressure.

2. Materials and Methods

2.1 Materials and Equipment

Glucose oxidase from *A. Niger*, Sodium Acetate, L-Tryptophan, and Acetic acid was purchased from Sigma Aldrich.

The HHP system (Figure 1) was composed of a high-pressure micro pump (model MP5), a high-pressure optical cell (model U103) with sapphire windows, and a pump controller (MP5 micropump control unit) from Unipress Equipment (Warsaw, Poland). Fluorescence measurements were done by placing the HHP reactor inside a Cary Eclipse fluorescence spectrophotometer from Agilent (Santa Clara, CA, USA). The excitation wavelength was set to 280 nm, and emission spectra were collected from 300 nm to 400 nm. Inside the spectrophotometer, the high-pressure cell sat on top of an XY2-axis manual linear stage 60x60 mm trimming bearing tuning platform sliding table Misumi (Schaumburg, IL). Water baths,

Isotemp 6200 H11 (20 °C – 80 °C) and Isotemp 6200 R28 (10 °C) from Fischer Scientific (Pittsburg, PA, USA) fed water into the jacket of the high-pressure reactor controlling the temperature. The high-pressure reactor was filled with silicone oil to act as a pressure medium. The water flow from the water baths was controlled by a pair of Sirai Z110A solenoid pinch values (Busseri, Italy). A type K thermocouple inserted through the side of the reactor monitored the temperature within the reactor. LabVIEW recorded program process time, temperature, pressure, and the solenoid valves are controlled with data acquisition boards (NI cDAQ 9174) from National Instruments (Austin, TX, USA).

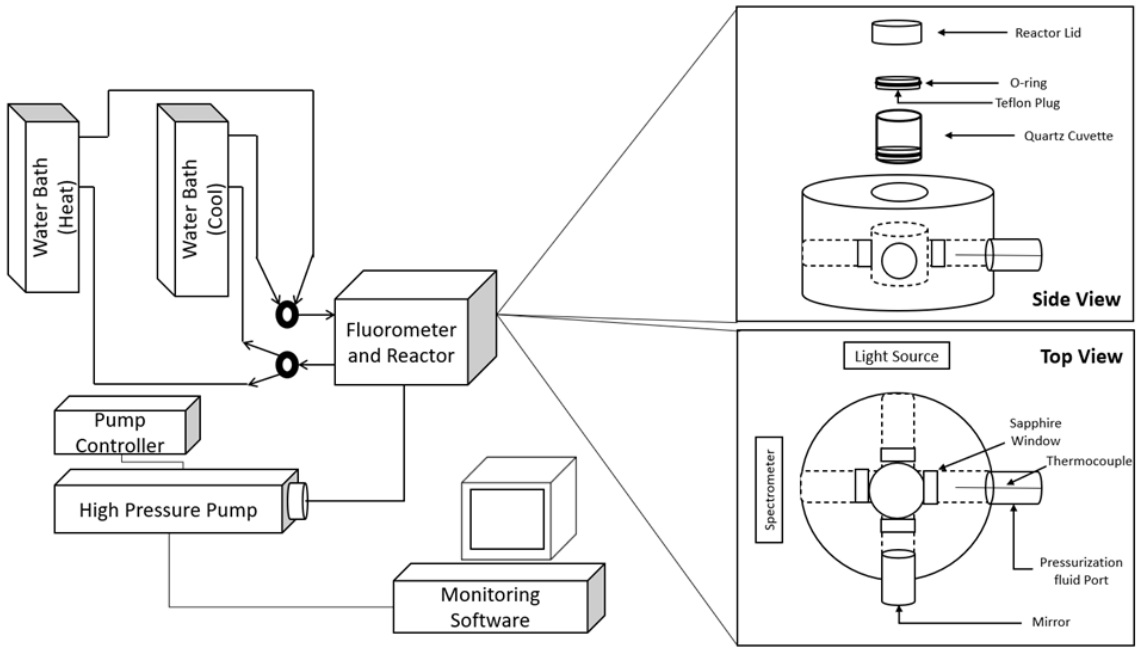

Figure 3.1. Simple graphic depiction of high hydrostatic pressure and fluorescence equipment

2.2 Methods

2.2.1 High-Pressure Differential Scanning Fluorescence System Design and Experimental Setups.

2.1.1.1 Design Basics

The basic design concept required a fluorometer able to quantify intrinsic fluorescence (excitation at 280 nm and emission at 300 nm – 400 nm) continuously, a high-pressure cell with optical windows at 90° to hold the enzyme solution, a high-pressure pump that pressurizes the sample, and a temperature control system that allows ramping the temperature at a constant rate.

2.1.1.1 Setup #1 Halogen-deuterium lamp and fiber optic fluorescence spectrometer connected to the high-pressure optical cell.

High-pressure system used in this, and other prototypes proposed here consisted of a piston pump (model MP5), a high-pressure optical cell (model U103, Figure 1) with sapphire windows, and a pump controller (MP5 micropump control unit) from Unipress Equipment (Warsaw, Poland). Fluorometer consisted of a DH-2000 BAL for the light source and a model USB2000+ spectrometer from Ocean Optics (Orlando, FL). The light source, optical cell, and spectrometer were connected using fiber optic cables (Extreme Solarization Resistant Optical Fiber) attached to the high-pressure cell with collimating lenses (74-UV Collimating Lens) from Ocean Optics (Orlando, FL). The success of the fluorescence reading was determined by comparing the readings from inside the high-pressure cell to the same fluorometer setup but instead using a 1 cm cuvette holder (CUV) from Ocean Optics (Orlando, FL).

The first prototype did not record fluorescence readings for GOx (Figure 2). The GOx sample held in the 1 cm cuvette holder (Figure 2B) had a clear peak ~300 au between 300-400

nm which is expected range of tryptophan fluorescence using an excitation wavelength of 280 nm.[208] While the GOx sample held in the high-pressure cell did not display a noticeable peak between 300-400 nm and appears to have a large artifact between 400 – 900 nm (Figure 2A). It is hypothesized that the difference in fluorescence intensity can be attributed to the increase in pathlength of the light needed to travel outside of the fiber optic cables when using the high-pressure cell (10 cm) vs the 1 cm cuvette holder (2 cm) (figure 3). One way to combat this could be switching from the DH-2000 BAL, which uses a combination of deuterium and halogen lightbulbs as the light source, a more intense light source light source. LED and Xenon light sources were investigated for new light sources that can provide up to 200 times more intense signal at 280 nm compared to the DH-2000 BAL.

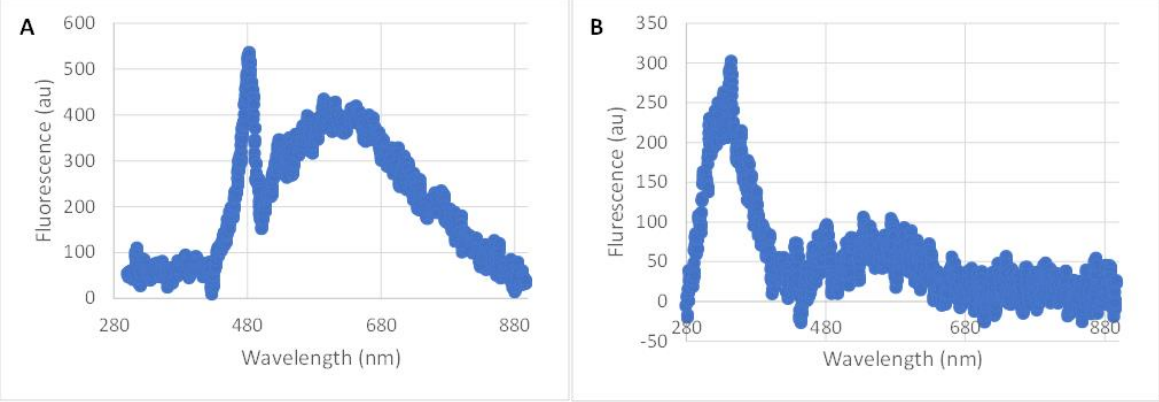

Figure 3.2. Fluorescence intensity of GODx from prototype 1 using ex 280 nm and em 280 – 900 nm A) Sample held and read in a high-pressure cell, and B) Sample held and read in a 1 cm cuvette holder

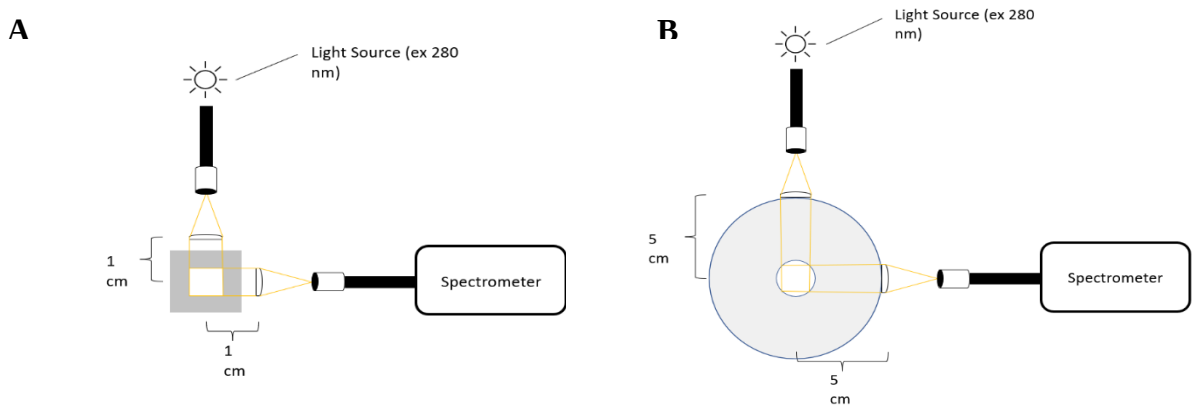

Figure 3.3. Light pathlength from light source to the spectrometer A) Sample held in a l cm cuvette holder B) Sample held in high-pressure cell

2.1.1.2 Setup #2 Fiber optic interface of a Cary fluorescence spectrophotometer to the high-pressure optical cell

Switching to a more powerful light source required switching the fluorometer setup from the one used in prototype #1 to a Cary Eclipse fluorescence spectrophotometer from Agilent (Santa Clara, CA, USA). The Cary Eclipse fluorescence spectrophotometer was not compatible out of the box with fiber optic cables. The fluorimeter needed to be modified to focus the xenon light source beam to the high-pressure HP cell and from the HP cell to the detector. Using the stand from the fluorimeter as a base, several 3D-printed attachments were created via the University of Georgia Makerspace to carry the light (Figure 4). The attachments transmitted the light from the light source to the high-pressure cell. The light source produced absorbance readings (data not shown), but a fluorescence reading could not be observed. The idea of inserting the high-pressure cell into the fluorometer would be explored.

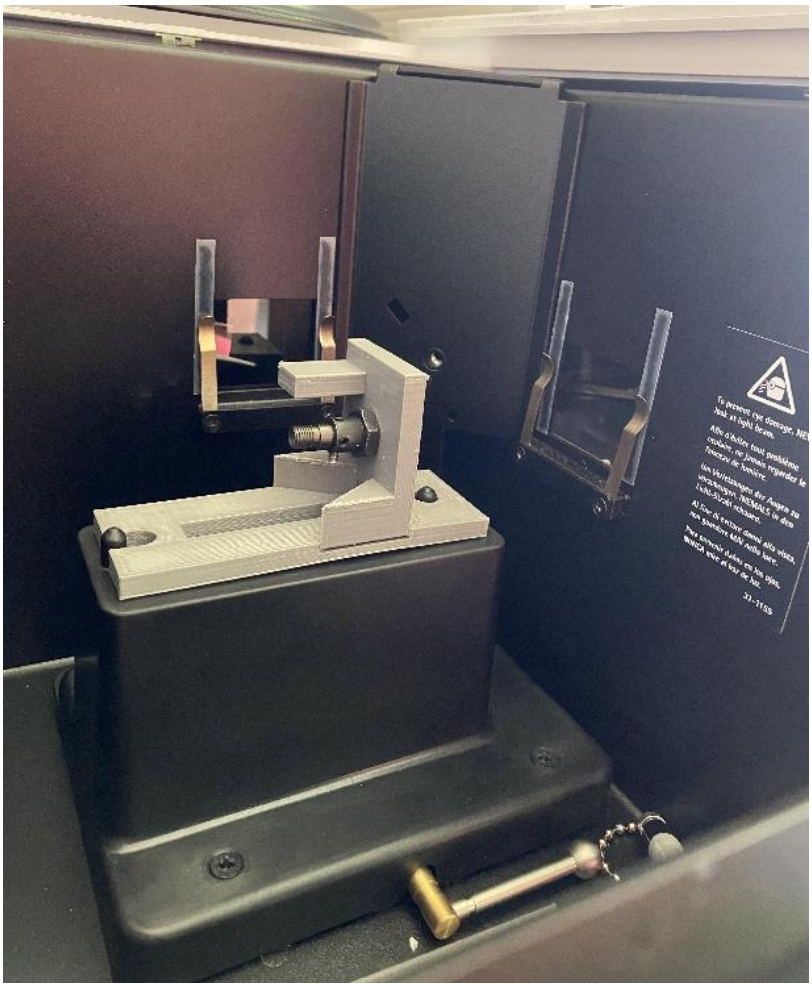

Figure 3.4. Cary Eclipse Fluorescence Spectrophotometer base with 3D printed fiber optic attachment

2.1.1.3 Setup #3. Placement of the high-pressure optical cell inside a Cary fluorescence spectrophotometer.

Inserting the high-pressure cell directly into the chamber of the fluorimeter required creating a stand that was sturdy enough to support the weight of the high-pressure cell while keeping the cell in the same spot to record measurements without variation. Originally, a 3D-printed stand was created to support the high-pressure cell. This prototype was able to measure the fluorescence from the high-pressure cell. Unfortunately, while the stand could keep the cell at a constant place on the z-axis, the 3D printed stand could not maintain a constant position on the x- and y-axis. To address this issue, an XYZ-axis manual linear stage 60 x 60 mm trimming bearing tuning platform sliding table Misumi (Schaumburg, IL) was used as the new stand for the high-pressure cell. The tuning platform allowed control over the x-, y-, and z-axis, allowing the cell to be consistently placed in the optimal location. An experiment was conducted measuring 1mM tryptophan fluorescence to determine the optimal position of the optical cell in the fluorometer (Figure 5). The cell position was locked in place at the position of maximal fluorescence for the remainder of this study.

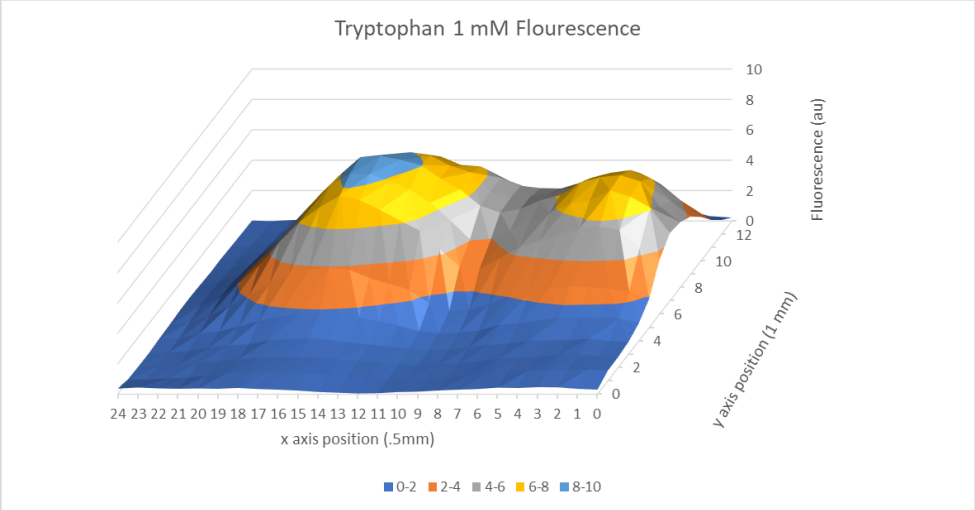

Figure 3.5. Tryptophan fluorescence vs xyz- axis location

2.2.2 Sample Preparation and Treatment Conditions

Glucose oxidase from *A. niger* was diluted to 10 $mg \cdot mL^{-1}$ in a 50 mM sodium acetate buffer pH 5.1 and placed on ice when not used. Aliquots of ~0.5 mL of glucose oxidase were placed inside a cylindrical quartz cuvette, capped, and immediately put into the high-pressure reactor. The reactor was kept at 10 °C by running cold water from the cold-water bath. Differential scanning fluorimetry experiments were performed at pressures 0.1, 50, 100, 150, 200, 300, and 400 MPa, using a linear temperature ramp of 20 – 90 °C at a scan rate of 1 $°C \cdot min^{-1}$. Intrinsic fluorescence was scanned every minute (($\lambda_{excitation}$ = 280 nm, $\lambda_{emission}$ = 300 – 400 nm). A blank was taken at 10 °C and 0.1 MPa prior to pressurization and ramping of temperature. Every pressure was tested in triplicate using a completely randomized design. Because the intrinsic fluorescence spectra were noisy, data was fitted to a 3^{rd}-order polynomial, and the emission intensity was determined from the regression calculation. Emission intensity curves were fitted to a sigmoid function. Chopping off data pre- and post-transition where the fluorescence started to decay after reaching a maximum. Statistical analysis was completed using GraphPad Prism (San Diego, CA) using analysis of variance (ANOVA) and Tukey's pairwise comparison, α =0.05.

3. Results and Discussion

The effect of temperature on fluorescence intensity at pressures 0.1 – 300 MPa was recorded in Figure 6. As the temperature increased (20 – ~60 °C), there was a slight decrease in fluorescence intensity until it experienced a sigmoidal curve (~60 – 80°C), after which the fluorescence intensity decreased again for all pressures. There was a decrease in the peak fluorescence intensity as pressure increased from atmospheric pressure to 300 MPa. As the temperature increased, there was a slight decrease in the fluorescence intensity but not an

apparent protein unfolding transition, which was reported to occur at 61.4 °C[86] at atmospheric pressure. Pressure's effect on the transition temperature is shown in Table 1 and Figure 7. A sigmoidal transition occurred at 69.7 ± 0. 5 °C at atmospheric pressure. This sigmoidal transition increased with pressure between 0.1 and 100 MPa to 76.0 ± 0. 2 °C. As pressure increased from 100 MPa to 300 MPa, no statistical difference ($p < 0.05$) was observed. At 400 MPa, the signal became too noisy to fit a sigmoidal curve and thus could not be used to calculate the T'. A substantial increase with a sigmoidal transition occurred at 69.7 ± 0. 5 °C, suggesting protein aggregation formation. Then, a decrease in fluorescence occurred, which can be attributed to either light backscattering and/or precipitation of large protein aggregates.

Table 3.1. Thermal transition temperature, T' for each pressure treatment

Pressure (MPa)	T' (°C)
0.1	69.7 ± 0.5[a]
50	73.4 ± 0.6[b]
100	76.0 ± 0.2[c]
150	76.7 ± 0.2[c]
200	78.0 ± 0.3[c]
300	78.3 ± 1.1[c]

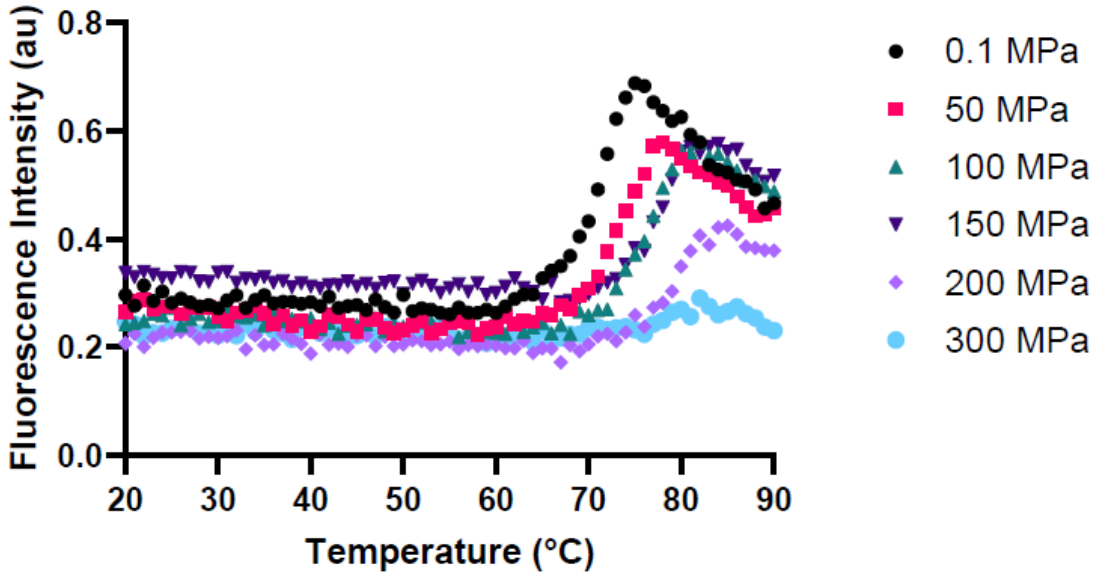

Figure 3.6. Fluorescence intensity vs temperature plot. Intrinsic fluorescence of tryptophan plotted against temperature from 20 – 90 C. One fluorescence intensity is shown per pressure.

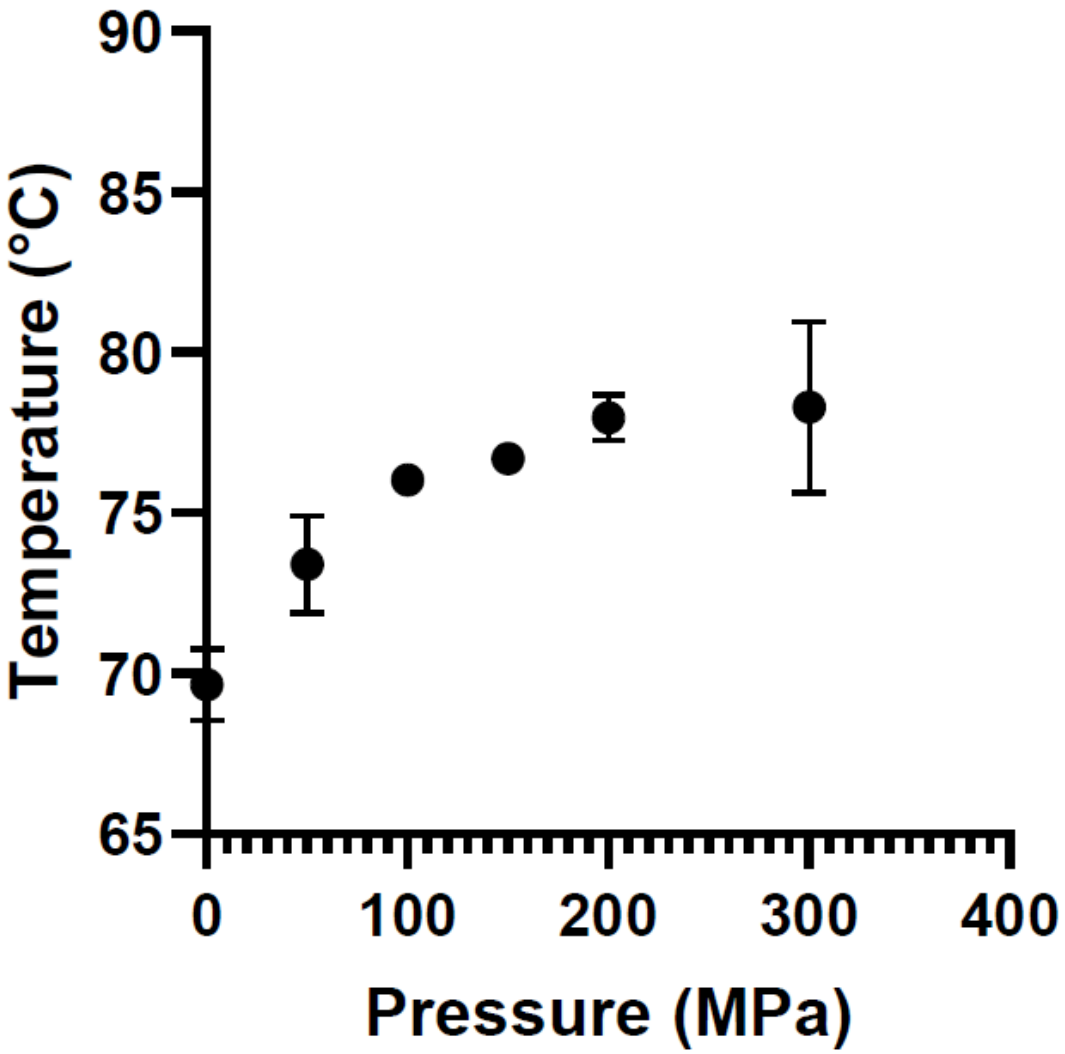

Figure 3.7. Transition temperature vs pressure plot. T' is measured from the first derivative of fluorescence intensity. T' Error bars represent a 95% confidence interval determined by Tukey's test.

Our results varied from previous studies and reported a reported a T_m of 69.7 °C for glucose oxidase atmospheric pressure. Glucose oxidase had a T_m of 61.4 °C[86] and 55.8 °C.[209] These previous studies were conducted in pH 7.1 – 7.2 phosphate buffers versus ours in acetate buffer with a pH of 5.1. With a pI of 4.2 and an optimal pH of 5.5, we expect increasing the pH outside the optimal zone to decrease T_m.[193,210] Studies have reported the influence of scan rate, pH, buffer type and concentration, and salt concentration.[211-213]

Our results suggest that glucose oxidase experiences conformational changes during heating, such as the formation of aggregates. Similarly, Sattari[214] and Gouda[215] found conformational changes that occur when glucose oxidase is heated above 60°C cause exposure of hydrophobic surfaces, resulting in the formation of aggregates. The thermal unfolding of glucose oxidase consists of the protein losing part of the secondary and tertiary structure, resulting in protein unfolding. This is an irreversible transition from the native protein to the compact unfolded form. Non-specific aggregation is favored because of the hydrophobic interactions of the side chains[209,216]

Table 1 and Figure 7 show that T' shifted from 69.7 ± 0.5 °C at atmospheric pressure to 76.0 ± 0.2°C an increase of ~6 °C. The T increase and the overall decrease in fluorescence intensity suggest that HHP reduces aggregation. Conformational changes or the formation of new molecular interactions that decrease the Gibbs free energy within the complex, increase the protein's thermal stability and T'.[204] If pressure prevents aggregation of the protein, the concentration of unfolded protein is greater than at atmospheric pressure. Therefore, to maintain equilibrium, pressure also opposes the unfolding of the protein.

Similarly to the increase in T' with pressure, the rate constant of inactivation, $k_{inact,}$ of glucose oxidase, decreased as pressure was increased, increasing thermal stabilization. From 0.1 MPa up

to at least 180 MPa from 58.8 °C – 80.0 °C. k_{inact} was the lowest at temperatures 63.8 - 80 C between 180 – 300 MPa, while at 58.8 °C k_{inact} was lowest between 180 – 240 MPa. The pressure that correspond with the lowest k_{inact} are the pressure with maximum enzyme stabilization. Halablipour et al. used Eyring equation/plot to calculate glucose oxidase stabilization and inactivation based on activation volume. Between 0.1 and 180 MPa, there was an observed decrease in the rate constant of inactivation. This deviated from a linear regression between 180 and 300 MPa, indicating a pressure-based shift from stabilization to inactivation.[86]

4. Conclusion

This chapter describes the design and construction of an HHP system to measure intrinsic fluorescence. The system was then used to measure the effect of HHP on the intrinsic fluorescence of glucose oxidase. Our results suggest that glucose oxidase experiences large conformational changes during heating, such as the formation of aggregates. The results demonstrate a positive shift in the T' of glucose oxidase as pressure increases from 0.1 to 300 MPa. In terms of equilibrium, pressure prevents protein aggregation and creates a positive shift in T'. These results confirm the stabilizing effects of pressure on GOx and suggest that partial unfolding of the protein is followed by aggregation.

CHAPTER 4

FINAL COMMENTS

Overview

Pressure's effect on enzyme stability differed for each enzyme. An optimal pressure was determined for LOx and AsOx to be between 50 – 100 MPa, the enzymes experienced a 1.5- and 1.3-fold increase in stability, respectively. ClOx was stabilized up to at least 600 MPa with no observable max increasing enzyme stability 15.8-fold. While ChOx was partially denatured by pressure with no clear stabilization effect. There does not appear to be a clear association between optimal pressure and enzyme size, quaternary structure, or total enzyme cavity volume.

An HHP system was able to be designed and constructed to measure intrinsic fluorescence from atmospheric pressure to 300 MPa. Glucose oxidase experienced large conformational changes during heating, including the formation of aggregates. Transition temperature (T') increased by 6°C from atmospheric pressure to 100 MPa with no observed maximal T' in the observable pressure range. The positive shift in T' suggests that pressure decreases the extent of protein aggregation.

Future Work

Future work is required to understand the effect of HHP on enzyme stabilization. This study used a coarse estimate of the pseudo-first-order rate constant of inactivation using activity at treatment time 0 min and the residual activity after a 5 min treatment time for LOx, AsOx, ChOx, and ClOx While it was able to obtain an order of magnitude estimation of the activation volume of stabilization and inactivation, using additional treatment times would help to provide a

more accurate estimation of these values.

The results of this study determined that the optimal pressure for LOx and AsOx was between 50 – 100 MPa. While pressure stabilized ClOx up to 600 MPa it was a denaturant for ChOx. Perhaps testing additional pressures for LOx (25 MPa and 75 MPa), AsOx (25 MPa and 75 MPa), ClOx (+700 MPa) and ClOx (50 MPa, 150 MPa, 250 MPa and 350 MPa) a more exact look at HHP effect on thermostabilizing can be obtained. Potentially narrowing in on the optimal pressures of these enzymes.

Our residual activity findings allowed us to determine the effect of HHP on the thermal inactivation of the LOx, AsOx, ClOx and ChOx. While these results do allow us to explore the effect of pressure on thermal inactivation, it does not provide information on the structural changes that are occurring in our range of pressures. Further testing is required to understand better the structural changes undergoing LOx, AsOx, ClOx and ChOx. Analyses that would complement this research include but are not limited to fluorescence spectroscopy to characterize protein folding and conformational changes, nuclear magnetic resonance spectroscopy to study the changes in conformation, denaturation, and internal mobility, circular dichroism spectroscopy to examine the secondary structure and folding properties, Fourier transform spectroscopy to examine the secondary structure and local conformational changes, and SAXS to measure radius of gyration and folding states but were out of scope for this project.
HHP's effect on glucose oxidase thermal transition temperature was able to be determined using differential scanning fluorimetry and suggests that pressure prevents protein aggregation. Unfortunately, the experiments could not be conducted using LOx, AsOx, ClOx and ChOx due to the low signal and high noise. The future development of this method and modification of the equipment could allow for an increase in signal and a decrease in noise.

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