EVALUATION OF HIGH PRESSURE PROCESSING FOR IMPROVING QUALITY AND FUNCTIONALITY OF EGG PRODUCTS

By

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ABSTRACT

The main objective of this study was to evaluate the effect of high pressure treatments on liquid rheology, color, texture and functional properties of egg products, specifically egg white (EW), egg yolk (EY) and whole liquid egg (WLE). High pressure processing is a novel and innovative method for extending the shelf-life and enhancing quality of foods. Various experimental designs and statistical analyses were used to study the effect of high pressure on all three components – EW, EY and WLE all initially in the liquid state. Since the egg components transformed from the flowing liquid state to a viscous liquid to soft gel and finally to a solid gel structure with the increase in pressure severity, a single rheological technique was not suitable to study all of them. The liquid rheology was studied using conventional steady and dynamic shear viscometry, the viscous to semi-solid samples was assessed using a back-extrusion rheology and finally the solid gels were assessed using a texture profile analysis. These studies generally indicated the progressive increase in rheological properties (toward texture build-up) with an increasing severity of pressure treatment (higher pressure and higher treatment times). HP treatment caused significant changes in the viscoelastic properties and these changes correlated well with functional properties. It was observed that high pressure induces gelling in the egg components which is caused by crossover of viscous and elastic moduli. This phenomenon caused significant enhancement of functional properties like water holding capacity and foaming properties in egg components. HP treatment also resulted in desirable changes in color with enhancement of brightness and yellow color. As with heat processing, the egg white turned white following HP treatment.

Avidin is an anti-nutritional component present in egg. If not inactivated, it will bind biotin which is essential for the functioning of thyroid glands. Hence, it is essential that the avidin present in raw egg be inactivated prior to consumption of the egg. Heat treatment is usually used to inactivate avidin. In this study, HP treatment was evaluated and found to be more effective than heat for inactivating the anti-nutritive avidin.

The last part of the study was focused on the effect of HP treatment on the functional properties of egg components, especially the degree of hydrolysis and

antioxidant activity. These changes were mainly due to partial unfolding of proteins and exposition of buried peptide bonds which are responsible for antioxidant activity which leads to their enhancement. DPPH (1, 1-diphenyl-2 picrylhydrazyl) radical scavenging assay showed higher antioxidant activity in pressure treated sample than the control sample.

RESUME

L'objectif principal de cette étude était d'évaluer l'effet d'un traitement à haute pression (HP) sur la rhéologie liquide, la couleur, la texture et les propriétés fonctionnelles des ovoproduits, en particulier le blanc d'oeuf (BO), le jaune d'oeuf (JO) et l'œuf entier liquide (OEL). Le procédé de haute pression est une méthode nouvelle et innovatrice pour augmenter la durée de conservation et améliorer la qualité des aliments. Divers modèles expérimentaux et analyses statistiques ont été utilisés pour étudier l'effet des hautes pressions sur les trois produits d'oeufs – BO, JO et OEL, tous initialement à l'état liquide. Étant donné qu'avec l'augmentation de la pression les produits d'œufs se transforment d'un état liquide limpide à un état liquide plus visqueux, à un gel mou et enfin à une structure de gel solide, une technique rhéologique unique n'était pas appropriée pour tous les étudier. La rhéologie liquide a été étudiée en utilisant la method conventionnelle de viscosité de cisaillement dynamique et stationnaire, les échantillons visqueux à semi-solides ont été évalués à l'aide de la une rhéologie d'extrusion inversée et, enfin, les gels solides ont été évalués en utilisant une analyse du profil de texture. Ces études ont généralement indiqué l'augmentation progressive des propriétés rhéologiques (vers la texture plus rigide) avec l'augmentation de la pression émise (pression et temps de traitement plus élevés). Le traitement HP a provoqué des changements significatifs des propriétés viscoélastiques et ces changements sont en bonne corrélation avec les propriétés fonctionnelles. Il a été observé que la haute pression induit la gélification des ovoproduits qui est causée par croisement des modules visqueux et élastique. Ce phénomène a causé une amélioration significative des propriétés fonctionnelles telles que la capacité de rétention d'eau et les propriétés moussantes des ovoproduits. Le traitement HP a également entraîné des changements souhaitables dans la couleur avec l'amélioration de la luminosité et la couleur jaune. Comme avec le traitement thermique, le blanc d'oeuf est devenu opaque avec le traitement.

L'avidine est un composant antinutritionnel présent dans l'œuf. S'il n'est pas inactivé, il se liera à la biotine qui est essentielle pour le fonctionnement de la glande thyroïde. Par conséquent, il est essentiel que l'avidine présente dans l'œuf cru soit inactivée avant sa consommation. Le traitement thermique est généralement utilisé pour inactiver l'avidine. Dans cette étude, le traitement HP a été évalué et jugé être plus efficace que la chaleur pour inactiver l'avidine antinutritive.

La deuxième partie de l'étude était axée sur l'effet du traitement HP sur les propriétés fonctionnelles des produits d'oeufs, en particulier le degré d'hydrolyse et de l'activité antioxydante. Ces changements sont principalement dus à un dépliement partiel de protéines et à l'exposition des liaisons peptidiques responsables de l'activité antioxydante qui conduit à leur amélioration. Le test de réduction du radical stable DPPH (1, 1-diphényl-2 picrylhydrazyl) a montré une activité antioxydante plus élevée pour l'échantillon traité sous pression que pour l'échantillon de contrôle.

CONTRIBUTIONS OF AUTHORS

Several parts of the thesis research have been presented at international conferences and some manuscripts have been prepared for publication. Principally two authors have been involved in the thesis and their levels of contributions to the various articles are as follows:

Ajaypal Singh is the PhD candidate who, under the guidance of his supervisor, planned and conducted all the experiments, gathered and analyzed the results, and drafted all the manuscripts for scientific publications.

Dr. Hosahalli S. Ramaswamy is the thesis supervisor, under whose guidance the research plan was carried out, and who assisted the candidate in planning and conducting the research with his expertise in high pressure processing, rheology and texture analysis. He was also responsible for the final correcting, editing, reviewing and processing the manuscripts for publications.

Prabhjot Singh was summer student who has helped in antioxidant activity work and provided valuable discussion and inputs for Chapter 8.

LIST OF PUBLICATIONS AND PRESENTATIONS

Part of this thesis has been prepared as manuscripts for publications in refereed scientific journals:

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CHAPTER 1

INTRODUCTION

Egg has exceptional qualities as a food and is known for providing a high level of nutrition. It constitutes a unique source which can supply all essential amino acids, several vitamins and minerals, including vitamin A, riboflavin, folic acid, vitamin B_6, vitamin B_{12}, choline, iron, calcium, phosphorus and potassium. Egg proteins are well recognized in food industry for their whipping, gelling, foaming and emulsification characteristics, where the proteins act as foremost components in determining the functional properties. They are high in nutritive value, and thus act as potential host and carrier for pathogenic micro-organisms like *Salmonella enteritidis* (White et al., 2007). *Salmonella enteritidis* is responsible for more than 90% of food-borne *Salmonellosis* which occurs through shell eggs (White et al., 2007).

Currently, thermal treatment is used for pasteurization of eggs. Pasteurization of egg is done under mild conditions in order to prevent extensive denaturation of their proteins. Even the heating at 60ºC for 20-25 min can cause partial denaturation and coagulation resulting in adverse effect on the functional properties of the egg. High pressure processing (HPP) under mild conditions has ability to pasteurize the eggs by inactivating *Salmonella* without adversely affecting the functional characteristics of the egg (Hayashi, 1989; Ponce et al., 1999; Ahmed and Ramaswamy, 2003). HPP is an emerging alternative technology to commercial thermal processing. It can kill spoilage and pathogenic micro-organisms. Consumer trend towards natural flavor and taste of food has given advantage to investigate substitute food processing technologies in this area. This technology works on the isostatic rule which governs that pressure is instantaneously and uniformly transmitted throughout a sample. Therefore, in contrast to conventional thermal processing, HP treatments can be given independent of the sample size and shape (Rastogi et al., 2007). The technique offers a cutting edge over others by reducing the chances of post-process contamination, coagulation and the nutritional qualities are well retained due to the fact that the temperature can be kept quite low during processing (Knorr, 1996).

HPP has shown promise to inactivate spoilage causing micro-organisms and it works well in inactivation of enzymes (Alderton et al., 1976; Oxen and Knorr., 1993; Arroyo et al., 1997; Mussa et al., 1999; Denys et al., 2000) resulting in a product of desired quality. It has shown to cause a 7-$\log_{10}$ reduction of *S. enterica* serovar *enteritidis* in liquid whole eggs (Ponce et al., 1998). It can contribute towards improvement of the physico-chemical properties like color, gel characteristics and water holding capacity of the food systems (Johnston et al., 1993). However, the published information is lacking in many perspectives like effect of HPP on physico-chemical and functional properties and microbiological safety of different components of egg.

High pressure works on principle of instantaneous creation of pressure and its uniform action on food ingredients while pressurizing. It gives a direct advantage over thermal processing, where temperature gradient leads to a cold spot in the food to be processed. Adiabatic heating during compression results in quick heating and the subsequent decompression results in its rapid cooling that would reduce overall processing time required for pasteurization in comparsion to thermal treatment, thus achieving lethality in minimum time and in turn promotes better quality retention. Thus, HP processing can be used as a special technique for rapid "heating and subsequent cooling" of packaged foods, which should provide quality advantage for the process (Shao et al., 2010). On similar note, HP can cause partial unfolding of proteins that can improve the functional and physical properties of the egg components. The changes in egg components obtained after high pressure treatment will help to correlate and further elucidate the change in protein functionality with structural changes.

The overall objective of this study was to develop the HPP as a complete process for pasteurization of egg and improve their functional, physico chemical and nutritional properties.

The specific objectives of this study were:

a) To characterize the effects of high pressure treatment on egg components as their structure evolve from liquid state to solid gel through semi viscous to semi-solid like materials using assortment of rheological and texture evaluation methods.

b) To characterize effect of high pressure processing on the anti-nutritional component, avidin (enzyme), with the objective of inactivating it through HP treatment.

c) To evaluate the impact of high pressure processing on the degree of hydrolysis and antioxidant activity of egg components. Conventional tryptic hydrolysis of egg white protein and the antioxidant activity of egg white protein and egg white hydrolysate were used as base condition.

d) Investigate the changes in viscoelastic and functional properties of egg components as function of increasing HP level and treatment time to better understand the basis of HP-induced viscoelasticity changes in solution and as well as in soft gels.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical perspectives of HP processing

The current food consumption trends indicate that the new millennium will be met by higher demand for better quality food product that are shelf stable, safe and additive free. The food industry has met these demands by developing a number of concept driven non thermal or mild thermal approaches to food processing of which high pressure technology has shown enormous potential. Therefore, much of the recent scientific research initiatives for the food industry are focused on non-thermal processing, with high pressure processing showing novel and diverse applications of the industry with commercial potential. (Alvarez et al., 2008; Knorr, 1996; Rastogi et al., 2007).

High Pressure Processing (HPP) is a method of food preservation by short time application of high pressure. It causes destruction of micro-organisms at room or low temperature, enzymes and even spore forming bacteria can be inactivated by pressure and thermal combinations and cause changes in conformation of biopolymers leading to changes in functionality and phase changes (Knorr, 1996). High pressure processing (HPP) is similar in concept to cold isostatic pressing of metals and ceramics, except that it demands much higher pressures, faster cycling, high capacity, and hygienic conditions (Zimmerman and Bergman, 1993; Mertens and Deplace, 1993).

HPP has been applied for many years to produce plastics, carbon graphite and ceramic product and has been known as potential food preservation techniques (Smelt, 1998). Further the applications of high pressure in various sectors -fruits and vegetables, dairy and meat processing have been dealt extensively. Hite (1899) was the first person to demonstrate that, spoilage of milk by micro-organism can be delayed by applying High Pressure. They expanded further their experiments from treating meat and milk products to fruits and vegetables by successfully preserving peaches and pears by pressure treating them at 400 MPa for 30 min at room temperature (Hite, 1899; Hite et al., 1914). Manufacturing and operation of high pressure machines at that time was difficult due to technical complexities. In 1905, W. Bridgeman was the first one to design a high

pressure unit with leak proof sealing ensuring that with sealing, the system always remained at higher pressure. This major change lead to evolution of changes that includes pioneering HP devices and further technical innovations resulting in whole new set of studies evaluating food materials under higher level of pressure.

Bridgman experiments were followed by Suzuki (1960) who reported the combined effect of pressure and temperature on the kinetics of protein denaturation deduced by turbidity. He calculated the thermodynamic functions in the activation process of denaturation from the relations of absolute reaction rates obtained from ovalbumin and carbonyl hemoglobin. Now with advances in food engineering area, high pressure machines are becoming available for general applications (Mertens and Deplace, 1993; Zimmerman and Berman, 1993). Commercially high pressure was first used in Japan in 1991 to sell high quality foods of low pH such as jellies, jams, juices, yogurt and salad dressings (Horie et al., 1991; Farkas, 1993; Zimmerman and Berman, 1993). This success was result of tie up between Ministry of Agriculture Japan, Forestry and Fisheries together with 21 food companies to set up industrial unit at University of Kyoto to develop HP process for use in the food industry (Johnston, 1992). The first high pressure processed foods were introduced in Japanese market in 1990 by Meidi-ya, manufacturer of jam, jellies, and sauces packaged and processed without heat (Thakur and Nelson,1998).

Other products treated by high pressure technique were fruit preparations, fruit juices, rice cakes in Japan; fruit juices in France and Portugal; and oysters and guacamole in the USA (Hugas et al., 2002). High pressure treatment can result in food products acquiring novel structure and texture, and hence can be used to develop new products (Okomato et al., 1990). In general, high pressure technology can substitute the use of chemical preservations and supplement the conventional thermal processing for reducing microbial load.

This technology has highly attracted research attention due to:

1. Changes in physical and functional properties of food systems.
2. Extending the keeping quality of product (Cheftel, 1995).

3. Anomalous phase transition of water under extreme pressure (e.g. lowering of freezing point with increasing pressures (Knorr, 1999).

Now, with tremendous technological advances in food engineering area, suitable high pressure processing equipments are becoming available. This development and better quality product demand by consumer have increased the commercial applications of HPP in the food industry and stimulated high pressure studies all over the world.

High pressure processing is a technology that potentially addresses most recent challenges faced by the food industry. It can assist the production of fresh food alongside convenience and profitability associated with shelf life extension (McClements et al., 2001). Although HPP has not been homogenous throughout the food industry but this technology has already become a commercially implemented technology spreading from Japan to America, followed by Europe. HPP can be applied to a range of different foods, including juices and beverages, fruits and vegetables, meat-based products (cooked and dry ham), fish and pre-cooked dishes, with meat and vegetables being the most popular applications. There are plenty of European companies presently employing this technology and some of them include: orange juice by UltiFruit®; sliced ham by Pernod Ricard Company, France; (Sliced Ham by Espuña, Spain; and fruit jams by Solofruita, Italy (Urrutia- Benet, 2005).

High pressure processing techniques have also gained momentum in areas of food preservation outside of sterilization and pasteurization. The range of possibilities offered by combining high pressure with low temperatures (HPLT) has allowed the basis of a new field of HP food applications to be formed, such as pressure-supported freezing, thawing and subzero storage. Much work has been conducted in the development and optimization of HPLT processes, and new findings regarding the phase transitions of water, with consequential benefits for the food Industry, have recently been revealed (Urrutia-Benet et al., 2004).

HPP technology has made significant advances in last 30 years in the form of semi continous pilot scale units to successful commercially viable processes. HPP treatment of food is carried out using batch or semi continous process. HPP work has been extended to salsa, rice products, fish, poultry products and ready to eat meats. HPP

treatment can provide fresh like taste, minimal processing and high quality convenient products with an extended shelf life.

Nowadays there are considerable amount of scientific publications on HPP related topics produced per year and the number keeps on rising.

2.2 Principles of HPP

HPP has emerged as the most innovative non-thermal food processing technique during past few decades. The first report was in late seventeen century; H. Rogers killed bacteria by high pressure (Rogers, 1895). Hite (1899) performed the first most important work involving microbial inactivation in food science by using high pressure. HPP is based on principle of isostatic distribution and Le-Chatelier principle.

2.2.1 Isostatic principle

The isostatic principle indicates that pressure transmittance occurs in a uniform and quasi instantaneous manner. The pressurization process time is independent of the sample volume. When an aqueous medium is compressed, the compression energy E (joule) is equal to

$$E = \frac{2}{5} \times P \times C \times V_0 \qquad (2.1)$$

Where

P= Pressure (Pa)

C= compressibility of solution

V_0= Initial volume (m^3)

So energy required for compression of 1litre water is 19.2 KJ at 400 MPa as compared to 20.9 KJ for heating one litre of water from 20 to 25°C. The covalent bonds of food constituents are less affected than weak interactions due to low energy levels involved in pressure processing.

2.2.2 Le Chatelier principle

The Le Chatelier principle describes the effect of pressure on the basis of absolute reaction rate theory. It states that, if a system at equilibrium experiences a change in concentration, temperature, volume, or partial pressure, then the equilibrium shifts to counteract the imposed change and a new equilibrium is established. Most biochemical reactions cause change in volume. So, biochemical processes are influenced by pressure application. Overall volume changes favour the disruption of hydrophobic bonds and dissociation of ionic interactions. Hydrogen bond formation is favoured while covalent bonds are not disrupted by high pressure.

2.3. Advantages of using HPP

The key advantages of HP processing are as follows:

1. Food processing can be done at ambient or lower temperature with help of HPP.
2. It causes instant transmittance of pressure throughout product, irrespective of size and shape, thus making size reduction optimal, which can be a great advantage.
3. It causes death of micro-organisms without any use of heat and preservatives/additives, therefore improving the quality of food.
4. It can be used to create food with novel functional properties.

The impact of high pressure on micro-organisms and proteins/enzymes is same as that of high temperature. High pressure causes transmittance of pressure rapidly and uniformly throughout the food. Therefore, the problems of spatial variations in preservation treatments associated with heat, microwave or radiations are not visible in pressure treated foods. By applying the pressure, the temperature of the liquid component of food increases by approximately 3°C per 100 MPa. The temperature rise is even greater (8-9°C /100 MPa); when the food contains fat such as butter or cream in high proportion (Rasanayagam et al., 2003). After a holding period, the foods cool down to their original temperature on decompression, if no heat is lost to (or gained from) the

walls of the pressure vessel. The temperature during the pressure holding period can change depending on the heat transfer rate across the walls of the pressure vessel, so desired temperature should be there for achieving truly isothermal conditions. When foods with empty spaces or voids are treated with high pressure, it can cause structural changes in fragile food containing entrapped air such as strawberries or lettuce.

Softening of cell and cell serum loss may result from cell deformation and cell damage. pH can be changed by using compression. Heremans (1995) indicated that with increase in pressure, lowering of pH in apple juice by 0.2 units per 100 MPa occurs. From thermodynamics' point of view, pressure has far reaching effects on conformation of macromolecules, transition temperature of lipids and water, and a number of chemical reactions (Cheftel, 1995; Tauscher, 1995).

Phenomena that result in decrease in volume are enhanced by pressure, and vice-versa (principle of Le-Chatelier). Thus under pressure, equilibrium of reactions are shifted more towards the most compact state, and reaction rate constant is increased or decreased, depending on whether Activation ($Complex_{vol}$ - $Reactants_{vol}$) is negative or positive. The low levels of energy utilised in pressure processing may explain why covalent bonds of food constituent are usually less affected then weak interactions. Due to this action, HPP can cause changes in volume. High pressures also control enzymatic reactions. The high pressure on protein /enzyme is reversible unlike with temperature, in range of 100-400 MPa and it is due to association and sub unit dissociation process and conformational changes (Morild, 1981).

2.4 Effect of high pressure on food constituents

2.4.1 Water

Water is a major constituent in most foods and high pressure processing markedly effects water. Water cannot be compressed at normal pressure, but it is partially compressible at high pressure. The water can be compressed upto 4% at 100 MPa and 15% at 600 MPa at 22°C. Foods with high water ratio will show similar kind of compressibility to water. Many physicochemical properties of water are reversibly

modified under pressure. Compression of water causes increase in temperature of water by 2-3°C per 100 MPa (Bridgeman, 1912).

Pressure can increase the ionic product [H+ OH-] of water. It can increase from 10-100 folds with application of 100 MPa pressure. The positive and negative charges are separated under pressure by a driving force called electrostriction. Water molecules rearrange in more compact manner with smaller total volume around electric charges, due to H-bonding and dipole-dipole interactions. The reaction $H_2O = H^+ + OH^-$ causes volume decrease of 21.3 mL per mole at 25°C (Kauzmann et al., 1962). Thus, pH of water, weak acids and several buffers decrease by 0.2-0.5 pH units per 100 MPa.

2.4.2 Proteins

Proteins are large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. High pressure causes denaturation of proteins depending on protein type, processing conditions and applied pressure level. Proteins can dissolve or precipitate on application of high pressure. These changes are reversible when pressure applied is in range of 100-300 MPa and irreversible when pressure level applied is higher than 300 MPa.

The destruction of hydrophobic and ion pair bonds, and unfolding of molecules is called denaturation. At high pressure, oligomeric proteins tend to undergo proteolysis. Monomeric proteins do not show any vulnerability to proteolysis with increase in pressure (Thakur and Nelson, 1998). High pressure causes rupturing of non-covalent interactions within protein molecules and further cause's reformation of inter and intra molecular bonds. Different types of interactions are responsible for secondary, tertiary and quaternary structure of proteins. The quaternary structure is mainly held by hydrophobic interactions that are sensitive to pressure. The tertiary and secondary structures of proteins can be significantly modified at pressures above 200 MPa. Final changes in conformation after HPP denaturation can cause full or partial unfolding of polypeptide structure which eventually results in exposure of peptides that can enhance antioxidant activity (Messens et al., 1997). Denaturation is complex process involving

intermediate forms leading to multiple denatured products. Secondary structure can even show irreversible denaturation at very high pressure above 700 MPa, leading to irreversible denaturation (Balny and Masson, 1993). When pressure increases to 100 MPa, temperature of denaturation of protein increases, whereas at higher temperature, temperature of denaturation usually decreases causing elliptical phase diagram of denatured proteins. At high pressure, proteins denature usually at room temperature than at higher temperatures. Pressure and temperature show antagonistic behavior at molecular level by following principle of microscopic ordering, which says that increase in pressure at constant temperature leads to an ordering of molecules or a decrease in the entropy of the system. An interpretation of reaction and activation volume is usually given in terms of intrinsic and solvent contributions. Intrinsic contributions may occur as a consequence of changes in free volume due to the packing density and/or the formation or breaking of covalent bonds. Temperature can induce irreversible changes as it breaks covalent bond and/or aggregation of unfolded protein.

High pressure has advantage for inducing protein denaturation as these effects are irreversible at pressure level <200 MPa and are not expected to reoccur. Temperature variations can lead to changes in both the volume and the thermal energy of protein but in contrast, at constant temperature under high pressure, the internal energy of the system is independent of pressure, and internal interactions are affected solely by the changes in the volumes of water structure and protein molecules. Denaturation is simply a two stated thermodynamic transition between two states of a protein. Interpretation of denaturation is difficult as the thermodynamic parameters are influenced by binding of the denatured molecules to multiple sites on a protein and this can change the binding of denaturant molecule to multiple sites on a protein and this binding changes the chemical potential of the protein.

Denaturation induced by pressure means that the volume occupied by the compact folded native conformation is larger than that of unfolded part. Protein unfolding is characterized by a negative molar volume of denaturation. The size of the protein hydration shell increases by attraction of new water molecules by the newly exposed surface amino acid residues but this increase is more than that compensated by the

negative contribution from the disruption of electrostatic and hydrophobic interactions and disappearance of voids in the protein not accessible to solvent molecules (Mozhaev et al., 1996).

2.4.3 Enzymes

Enzymes are a class of proteins and have a 3-d configuration of molecules. Their biological activities arise from their active sites. Protein denaturation can cause loss of activity, or change the functionality of enzymes (Tsou, 1986). Enzymes activity can also be influenced by pressure induced decompartmentalization (Gomes and Ledward, 1996). Since protein denaturation is associated with conformational changes, it can change the functionality of the enzyme (e.g. increase or loss of biological activity, change in substrate specificity). Effects of high pressure processing on enzymes can be divided in two classes: In first class, we can take pressure which is used to activate some enzymes in food to improve food quality (Jolibert et al., 1994). On other hand, undesirable enzymes in food can be inactivated using high pressure level. In regard to pressure inactivation, Miyagawa et al. (1964) explained that there are four distinguished groups of enzymes, based on recovery and loss of activity;

1. Completely and irreversibly inactivated
2. Completely and reversibly inactivated
3. Incompletely and irreversibly inactivated
4. Incompletely and reversibly inactivated

In an intact food tissue, enzymes and substrates are separated by compartments and pressure can induce membrane damage resulting in leakage of enzyme and substrate which can cause enzymes to contact the substrate. Pressure can cause enzyme inactivation, but there is a minimum level of pressure below which there is no action on enzyme. This pressure inactivation range is dependent on pH, medium, enzyme type, composition and temperature. This has been attributed to an enzyme portion that is irreversibly converted to the inactive form, while a fraction is converted to a very

pressure-resistant form. Upon pressure release, the pressure-resistant fraction reverts to the equilibrium state, while the irreversibly inactivated enzyme remains unchanged.

It has been found that high pressure enzyme inactivation can be improved by applying pressure cycles. Successive application of high pressure can result in higher inactivation of many enzymes (trypsin, chymotrypsinogen and pepsin). For trypsin and chymotrypsinogen, it has been reported that successive pressure treatments result in a higher degree of inactivation only when pressures above the minimum level required for their inactivation are applied (Ludikhuyze et al., 1997).

2.4.4 Vitamins

Food vitamins are inevitably and irreversibly damaged during thermal processing and vitamin contents in foodstuffs are closely interrelated to their nutritional quality (Noble and Gomez, 1962). Vitamins are highly sensitive to thermal treatments and other similar technologies which can cause great loss of vitamins by leaching. HPP is called cold treatment as it doesn't involve thermal cooking and is viable for processing of foods containing high amount of vitamins (Rovere et al., 1966). An HP treatment does not cause any significant loss of vitamin B_6 and B_1. Butz and Tauscher (1997) found that vitamin B_1 concentration was 1.475 and 1.468 μg/mL respectively in untreated and in sample treated at 600MPa/30 min/20°C. In similar way, HPP caused increase in vitamin concentration from 3.725 to 3.794μg/mL in model system after pressurization at 600 MPa/30 min/20°C.

Vitamins should be determined depending on fact that the amount present in food should be enough to complete nutritional requirement (Verhoeven, 1997). HPP doesn't affect the vitamin content as in strawberry nectar, ascorbic acid decreased only from 1129 to 1100 ppm after high pressure processing treatment (Balny et al., 1992). Strawberry coulis puree has high content of vitamin C and HP Treatment (400MPa/30 min/20ºC) caused 88.7% retention of total content. But on other hand, vitamin C content in coulis only had 67% retention after thermal treatment (120°C/20 min/0.1 MPa).

In other substrate like egg yolk, HP treatment caused insignificant effect on vitamin C concentrations even with treatments ranging from 400-1000 MPa, but vitamin

C concentration tend to decrease with increasing boiling time. It was found that there was small increase in vitamin C levels in egg yolk and egg yolk ascorbate after pressurization. HP treatment causes extractive effects from foods when they were pressurized at 200, 300 and 400 MPa/20°C/30 min, it caused ascorbate retention of 92.6, 101.34 and 102.6% respectively. Similar observations were made for thiamine retention in egg yolk as increase of 6.2 and 2.8% in thiamine retention after HP treatment of 600 and 800 MPa respectively for 30 min at 20ºC was found (Hayashi, 1989b).

2.5 High pressure processing of egg

2.5.1 Overview

Egg is highly nutritious as it is good source of proteins for human body. It is an excellent source of protein and a good source of 14 essential nutrients which have been detailed in Table 2.1. In addition, eggs contain vitamins and mineral elements which are vital for development of young and old people. Egg white proteins consist mainly of protein solution of ovomucin fiber, phosphoglucoprotein, ovotransferrin, ovomucin, lyzozyme and avidin (Stadelman and Cotterill, 1995). The egg yolk is dispersion of different kinds of particles suspended in protein solution containing: Phospovitin, Lipoviteline, Lipovitelenine, Liviteline and ovoviteline. It is a versatile food ingredient that can be used in number of food preparations. Egg is a low acid food with short shelf life, so it needs to be preserved using appropriate preservation method. Normally, thermal methods are used for its preservation, but it is not highly efficient as it causes reduction in nutritional and sensory quality of egg. So non thermal methods are used for its preservation, in which HPP is common as it maintains the shelf life of the product without causing any damage to its nutritional and sensory value because it doesn't affect the covalent bonds. Conformational changes in ovalbumin: protein component of egg white remains fairly stable when pressurized at 400 MPa, due to non covalent interactions and 4 disulfide bonds stabilizing three-dimensional structure of Ovalbumin (Hayakawa et al., 1992).

Canadian egg farmers produce about 420 million dozen "grade A" eggs, making up an average of 750 million dollars per annum (EFC, 2008). The cost of production is

1.70$ per dozen egg, which is double to that of American market. This occurs because the Canadian poultry industry operates on the principle of "Start Clean and Stay Clean" (CEMA 2002), which makes them expensive.

Table 2.1: Percentage of RDI* provided by one Canada Grade A large size egg

Vitamin A	8%
Vitamin D	2%
Vitamin E	6%
Thiamin	3%
Riboflavin	15%
Niacin	6%
Vitamin B6	2%
Folate	15%
Vitamin B12	30%
Pantothenic Acid	15%
Calcium	2%
Phosphorus	6%
Magnesium	2%
Iron	2%
Zinc	5%

*RDI= Recommended Dietary Intake

The Canadian Egg Marketing Agency follows a Hazard Analysis Critical Control Point (HACCP) based "Start Clean-Stay Clean^{TM} program" ensuring production of high quality, clean eggs that comply with internationally recognized safety standards. Canadian Food Inspection Agency (CFIA) is responsible for monitoring operations across Canada and takes random samples from egg grading and egg processing stations for assurance of hygienic practices.

Presently most of the commercially available liquid eggs are pasteurized using conventional heating method which is expensive and can cause damage to quality characteristics of egg. Pasteurization has gained a great commercial importance in recent years. Pasteurization is considered as the best solution to the *Salmonella enteritidis* problem in eggs. Current techniques for pasteurization of egg involve heating the egg white and egg yolk to 57.5 and 61.1˚C respectively for 2.5-3.5 minutes to ensure egg safety against *Salmonella* (FSIS-USDA 2006). This leads to the overheating of the egg white proteins (i.e. the egg white gets heated up more than the yolk, which is against the recommendations) resulting in denaturation and coagulation (Hou et al., 1996). This greatly affects the functional properties of the egg constituents. Therefore a process that can pasteurize eggs without use of heat would be the best alternative to solve this problem.

High pressure processing (HPP) is an alternative non-thermal preservation technique for pasteurization with much shorter processing time. The effect of high pressure on microorganisms and proteins/enzymes has been observed to be similar to that of high temperature, but its effect on quality characteristics is generally considered to be minimal. This effect has facilitated the laboratory findings to expanded full-scale production. Establishment of such high pressure pasteurization process requires data on pressure and temperature dependent inactivation kinetics of target pathogenic, spoilage or surrogate bacteria in the food being tested and its effect on quality parameters of food.

High pressure processing (HPP) at refrigerated, ambient or moderate heating temperature allows inactivation of pathogenic and spoilage causing 9+microorganisms in foods with fewer changes in texture, color and flavor as compared to conventional technologies (Hendrickx and Knorr, 2002; Torres et al., 2005; Cheftel et al., 1995). The compression generated on food during HPP treatment will increase the temperature of foods through adiabatic heating by approximately 3-9°C per 100MPa, depending on the amount of fat present in the food. Thus, adiabatic heating can help in achieving desired pasteurization or sterilization process. Compared with thermal processing, the main advantage of HPP is that it results in rapid heating to lethal levels during pressurization

and subsequent rapid cooling rate due to the quick depressurization. This feature will minimize food quality deterioration.

It was found that HPP produces softer and more elastic gels than heat-induced gels (Hayashi et al., 1989). This was first found when egg white and yolk gels were formed with high pressure level of 4000 to 10,000 kg/cm^2 and evaluated for their texture, protease susceptibility and nutrients. Egg white and yolk set to a stiff gel at or above 6000 and 4000 kg/cm^2, respectively. Taste and flavour of the pressure-induced gels was natural without a cooked taste and flavour. Subtilisin digestibility of the pressure-induced gels was compatible or superior to the heat-induced gels (Hayashi et al., 1989). In the former gels, no destruction of vitamins or amino acid residues and no formation of unusual compounds such as lysinoalanine were detected. Based on these results, it was proposed that HPP can be useful in food processing and preservation without any adverse effects on natural foods. Although the effects of pressure on proteins have already been studied for several decades, applied research on the application of high pressure to induce the denaturation, aggregation and gelation of food proteins did not become of significant interest until the late 1980s.

2.5.2 Protein modifications by HPP

The egg is one of the most nutritious and versatile of human foods. An average-sized egg weighs approximately 57 grams (about 2 ounces). Of this weight, the shell constitutes 11 percent; the white, 58 percent; and the yolk, 31 percent (Figure 2.1). Normally, these proportions do not vary appreciably for small or large eggs. Egg contains nutrients like proteins, carbohydrates, vitamins, minerals and phospholipids. Egg proteins provide desired nutritional and functional properties like foaming, emulsification, coagulation, color, thickening and binding, therefore it has diverse applications in many food products (Hayashi, 1992; Hsieh and Regenstein, 1992). Eggs can hold large quantity of air in the form of fine bubbles. These bubbles expand in cake mix and provide strength to air pockets. Pasteurization is required to avoid microbial contamination, but egg proteins are thermo sensitive and they can denature with pasteurization. To get

comprehensive understanding of this attribute, effect of processing methods on rheological behavior and protein modifications needs to be considered in detail.

2.5.2.1 Physiochemical modifications due to HPP

HPP can cause functional changes in food proteins. Some researchers have worked on the effect of HP on proteins at the structural level (Tedford and Schaschke, 2000; Galazka et al., 2000) and its effect on protein functionality (Ahmed and Ramaswamy, 2003) but very few studies have related these changes to commercial processing of foods. Studies dealing with changes in protein structure under heat induced gel formation are more abundant but changes caused in rheology due to high pressure applications are limited. These effects of HPP can be investigated by rheological analysis and will elucidate molecular basis of pressure induced changes in proteins functionality.

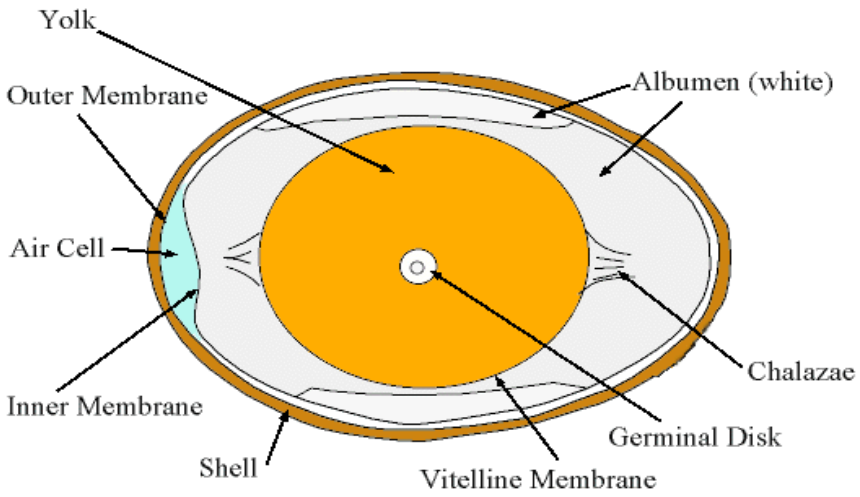


Figure 2.1- Components of an egg

(http://foodsafety.suencs.com/060909-egg-basics-for-the-consumer)

2.5.2.2 Protein interactions during HPP

Protein molecule is generally associated with physico-chemical and functional properties. Application of pressure can cause dissolution or precipitation of proteins. This does not involve breaking of covalent bonds (Ahmed et al., 2003). These changes are reversible when pressure applied is lower than 300 and irreversible in case of

pressure >300 MPa. It can cause destruction of hydrophobic and ion pair bonds and unfolding of molecules causing denaturation of proteins.

Numerous studies have been conducted on the effects of HP treatment on meat proteins, egg white, ovalbumin and soy proteins (Hayashi et al., 1989; Zhu et al., 2008; Alvarez et al., 2008). Still, additional experimental research on effect of HPP in food products is required to understand the potential of this technology in preservation of food products with minimal quality changes.

Proteins can dissolve, precipitate, aggregate, denaturate on application of high pressure. This technology can cause rupture of non-covalent interactions in protein molecules and further cause's reformation of inter and intra molecular bonds. Different types of interactions are responsible for integrity of secondary, tertiary and quaternary structure of proteins. The quaternary structure is mainly held by hydrophobic interactions which are sensitive to pressure applications. Even tertiary structure show considerable changes beyond 200 MPa pressure. Denaturation is a complex process involving intermediate forms leading to multiple denatured products. Secondary structure changes at very high pressure above 700 MPa lead to irreversible denaturation (Balny and Masson, 1993). When pressure increases to 100 MPa, temperature of denaturation of protein increases, whereas at higher pressure, temperature of denaturation usually decreases causing elliptical phase diagram of denatured proteins. At high pressure, proteins denature usually at room temperature rather than at higher temperatures.

HPP has shown great effect in restricting enzyme activity of fruit and vegetables juices and milk. Pectin Methyl Esterase (PME) activity was evaluated for single strength (pH 3.7 and 11.4 °B) and concentrated (pH 3.5 and 42 °B) HP treated orange juice. For both juices, the effect of all three main factors and their interactions (pressure level, cycle and holding time) were found to be statistically significant ($p<0.05$) (Basak and Ramaswamy, 1996).

Riahi and Ramaswamy (2004) explored high-pressure (HP) inactivation kinetics of PME in apple juice. The residual enzyme activity at pH 7.5 (30°C) was determined using titration method. The effect of pressure level and pressure holding time on enzyme inactivation were reported to be highly significant ($p < 0.05$). Inactivation kinetics were

evaluated on the basis of a dual effect model involving a pressure pulse effect and a first-order rate model, and the pressure sensitivity of rate constants was modeled using the z-value concept.

Pandey and Ramaswamy (2004) applied HP treatment to milk at pressure level of 300–400 MPa and evaluated lipoprotein lipase and γ-glutamyl transferase activity. Short time pressure caused enhancement in the activity of both enzymes, and for lipase, there was no inactivation during the entire pressure holding time (up to 100 min). With γ-glutamyl transferase, the extent of enhancement in activity was pressure level dependent, with lower pressure resulting in a greater enhancement. Furthermore, longer pressure treatment times resulted in inactivation of γ-glutamyl transferase, following a first order kinetic model (Pandey and Ramaswamy, 2004).

2.5.3 Rheology of eggs

2.5.3.1 Flow rheology: Rheology is defined as deformation and flow of raw material, intermediate and final products. It focuses on the relationship between force acting on a substance and its resulting deformation. Rheological tests helps in attaining knowledge on the structure of the food and its macromolecular elements, as well as assessing the textural attributes of the product prior to its processing, and based on these predictions, the food processing will be oriented towards achieving a final product with the characteristics that have proven desirable for the consumer. In the liquid form, even the viscous fluids tend to deform continuously under the effect of applied stress and can be categorized as either Newtonian or non-Newtonian fluids and are characterized mostly using a power law model:

$$\sigma = m\gamma^{n} \tag{2.2}$$

where σ is shear stress (Pa), γ is the shear rate (s^{-1}), m is the consistency coefficient ($Pa.s^{n}$), and n is flow behavior index.

2.5.3.1.1 Newtonian fluids

A Newtonian fluid is a fluid whose stress versus strain rate curve is linear and passes through the origin. It is the fluid which continues to flow, irrespective of the forces acting on it. Water is Newtonian fluid because it continues to represent fluid properties, it doesn't matter how fast it is stirred or mixed. Gases, oils, water and most liquids that contain more than 90% water, such as tea, coffee, beer, carbonated beverages, fruit juices and milk show Newtonian behavior (Barnes et al., 1989).

2.5.3.1.2 Non-Newtonian fluids

Fluids that do not follow Newton's law of viscosity are known as non-Newtonian fluids. The fluids showing non-linear slope of the shear stress versus shear rate graph are called non-newtonian fluids. For different shear rates, different viscosities are observed; therefore, apparent viscosity or a consistency term is used for non-Newtonian fluids. The variation of apparent viscosities with shear rates for different types of non-Newtonian fluids is presented is Figure 2.2.

2.5.3.1.2.1 Shear-thinning (pseudoplastic) fluids

In these types of fluids, viscosity tends to decrease with increase in shear rate. It results in temporary loss of viscosity, thus called pseudo plasticity. Typical examples of shear thinning fluids are paint and ink in a ball pen.

2.5.3.1.2.2 Shear-thickening fluids

In shear thickening fluids, rearrangement of microstructure occurs in such a way that the resistance to flow increases with shear rate. The internal friction and apparent viscosity increases with increase in shear rate. If the increase in the viscosity is accompanied with an increase in the volume, shear thickening fluids are called dilatant fluids.

High hydrostatic pressure (HHP) can cause denaturation of whole liquid egg (WLE) and albumen, thus changing the rheological behavior of egg samples. The

intensity of changes caused in food product is directly proportional to severity of pressure level used. Ahmed et al. (2003) analyzed effect of HPP ranging from 100-400 MPa on rheological characteristics of WLE, albumen, and yolk. It was found that all egg samples behaved as thixotropic fluids. The egg protein structure break-down was enhanced with pressure and it was found to be complete at 300 MPa for 30 min at 20°C. High pressure affected the protein structure of albumin and WLE; however, electrophoresis results exhibited that the protein coagulation was irreversible. The yolk behaved differently with pressure treatment. Similarly possibilities of a high pressure processing (HPP) combined process as an alternative to heat pasteurization of Liquid whole egg (LWE) (Monfort et al., 2012).

High pressure treatment criteria considering egg protein coagulation were investigated as first step of processing optimization for HPP of whole liquid egg (WLE). No coagulation was identified at pressure of 100 and 150 MPa until 3 600 s at 5 and 25 °C. Below the line of $\Delta W=5.0$ kJ/m^3, the rheological properties of processed WLE were comparable to that of fresh WLE and it is desirable to conduct high pressure treatment under this criteria (Knorr et al., 2003).

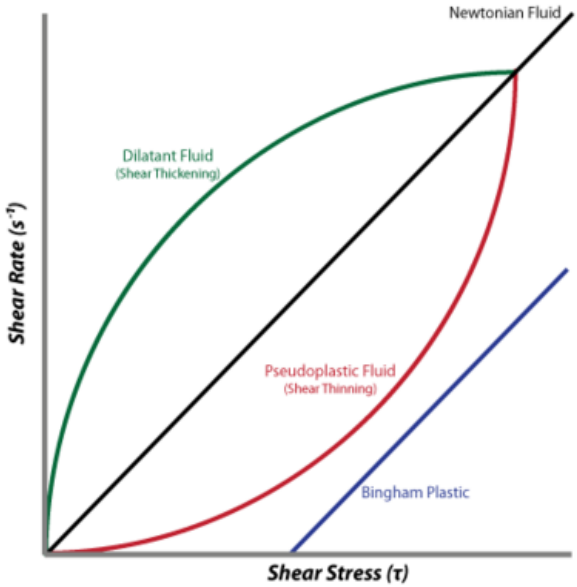


Figure 2.2: Non-Newtonian fluids (Barnes et al., 1989)

Similarly, the influence of high pressure and temperature on the rheological characteristics of glycomacropeptide (GMP) was studied. GMP dispersions at a concentration of 12.5% (w/w) were subjected to high pressure (100 to 400 MPa) and temperature (20 to 80°C) followed by rheological measurements at a shear rate ranged between 0 and $200s^{-1}$. Lower activation energy signified lesser molecular aggregation or unfolding of protein molecules during thermal treatment of GMP (Ahmed and Ramaswamy, 2004). In a similar study, it was found that longan juice with 0.15% xanthan treated with HPP gave optimal results for a fruit drink by viscoelastic determination. Colour parameters showed pressurised longan juice at 500 MPa was brighter and more transparent than fresh and other processed juices. Total phenols and antioxidant activity decreased significantly on pasteurisation, but were stable on pressurization (Chaikham et al., 2012). The effect of high pressure on structure of proteins can be evaluated using rheological and textural studies. Thus, in depth analysis and interpretation of rheological data needs to be supplemented by information about the underlying facts.

2.5.3.2 Small amplitude oscillatory methods

Food materials tend to have both viscous and elastic properties. These materials have an elastic and viscous component. As viscosity is resistance to flow, viscous material will lose energy in a loading cycle. Plastic deformation results in lost energy, which is not a characteristic of purely elastic material reaction. Viscoelastic material gives a hysteresis loop during stress-strain curve and area of the loop indicates energy lost during loading cycle.

Small amplitude oscillatory shear (SAOS) has been widely used due to its sensitivity to microstructure. Advantage of using SAOS is that it has an equation linking between the displacement sinusoid and the force sinusoid. This makes this technique particularly useful in the detection of viscoelastic properties (Taherian et al., 2008). It involves subjecting samples to small sinusoidal varying loads in which shear stress or strain is controlled.

Both the storage (G') and loss (G") moduli can be combined to give the tan δ which provides a ratio between the amount of energy stored and lost in a cycle, and hence a relationship between the elastic and viscous portions of the sample. The tan δ goes from zero to infinity, with higher values in Newtonian fluids and lowest in substances resembling hookean solids (Taherian et al., 2008).

2.5.4 Texture modifications by HPP

Texture is one of the most important quality characteristics of foods. Food texture can be evaluated by sensory or instrumental methods. Sensory methods need a taste panel containing trained panelists and it is hard to repeat the results. On the other hand, instrumental methods are less expensive and less time consuming as compared to sensory methods (Sahin and Sumnu, 2006). Many textural properties can be quantified using the Texture Profile Analysis (TPA), including hardness, (maximum force required to compress the sample), adhesiveness (work necessary to pull the compression anvil away from the sample), cohesiveness (study of how well the product withstands a second deformation relative to how it behaved under the first deformation), chewiness (calculated as product of gumminess and springiness (Length 1/Length 2), gumminess (calculated as product of hardness*cohesiveness (Area 2/Area 1), springiness (how well a product physically springs back after it has been deformed during the first compression) (Figure 2.3) (Gupta et al., 2007).

The geometry of the samples needs to be controlled, so the rule of thumb is to use samples of standard dimensions and pre-defined geometry to perform the tests, whereas force per unit area is called stress and the deformation per dimensional unit is called strain. It is especially important in the type of foods that cannot be cut in standard-size/shape pieces (i.e. lettuce and eggs). Only understanding how size and shape affect the deformability of the food is possible to separate the deformation measurements into differences due to changes in textural quality from those due to changes in size (Bourne, 1986).

HPP is known to cause considerable changes in food. Basak and Ramaswamy (1998) evaluated the effect of high pressure processing on texture of fruits and vegetables

by using pressure level of 100-400 MPa for 5-60 min in an isostatic press. Pressure had a dual effect on product texture characterized by an initial loss in texture, ascribed to the instantaneous pulse action of pressure, followed by a more gradual change as a result of pressure-hold. The extent of the initial loss and the subsequent partial recovery were pressure dependent with the former more prominent at higher pressures and the latter at lower pressures. The pressure treated samples were generally brighter in color somewhat resembling the appearance of mildly heat treated samples (Basak and Ramaswamy, 1998).

On the other hand with egg, it was found that pressure treatments above 500 MPa can cause coagulation and gelling (Bridgeman, 1914). Effect of high pressure and heat on texture of gels was compared. Egg yolk under pressure of 400 MPa for 30 min at 25°C forms gel, but 500 MPa is required for egg white to coagulate partially and 650 MPa and above for gelation to be complete. All egg parts have large amount of proteins and gel formation is responsible for denaturation of these proteins. On this basis, Juliano et al. (2006) found that high pressure processing can be used to improve texure and water retention improvement in scrambled egg patties. In simililar work, it was found that egg patties treated with pressure-temperature treatment of 700 MPa/105°C and 700 MPa/121°C did not produce gas or decompose after 6-month incubation, while control patties degraded after 1 week storage at 37°C (Juliano et al., 2007). Production of thermally sterilized egg based product with increased shelf life without losing the sensory and nutritional properties has been discussed in detail by Juliano et al. (2012). Grant et al. (1941) verified that coagulation by high pressure is associated with protein denaturation and showed that denaturation of proteins by pressure is not affecting any covalent bonds. In fact destruction of proteins is due to rearrangement of non-covalent bonds, such as hydrogen bonding, hydrophobic interactions, and ionic bonding contributing to tertiary structure. High pressure treatment is employed as an alternative to thermal treatment for reduction of microbial count, inactivation of spores and added advantage of vitamin potency and natural flavors of food. More recently, HPP has shown important role in improving protein functionality and gel texture of whey protein isolates (WPI) and on individual proteins found in WPI.

In a similar work, Atlantic salmon samples were frozen by different methods and subjected to different thawing treatments. Temperature and phase change behavior of fish samples were monitored during freezing and thawing. The phase change point of frozen salmon was lowered to −14°C, −19°C and −25°C for the HPT processes at 100, 150 and 200 MPa, respectively. These phase change temperatures were lower than for pure ice at the same pressures possibly due to the presence of solutes in salmon. Different freezing rates prior to thawing resulted in differences in drip loss in salmon samples, but they did not induce specific color and texture changes (Zhu et al., 2004).

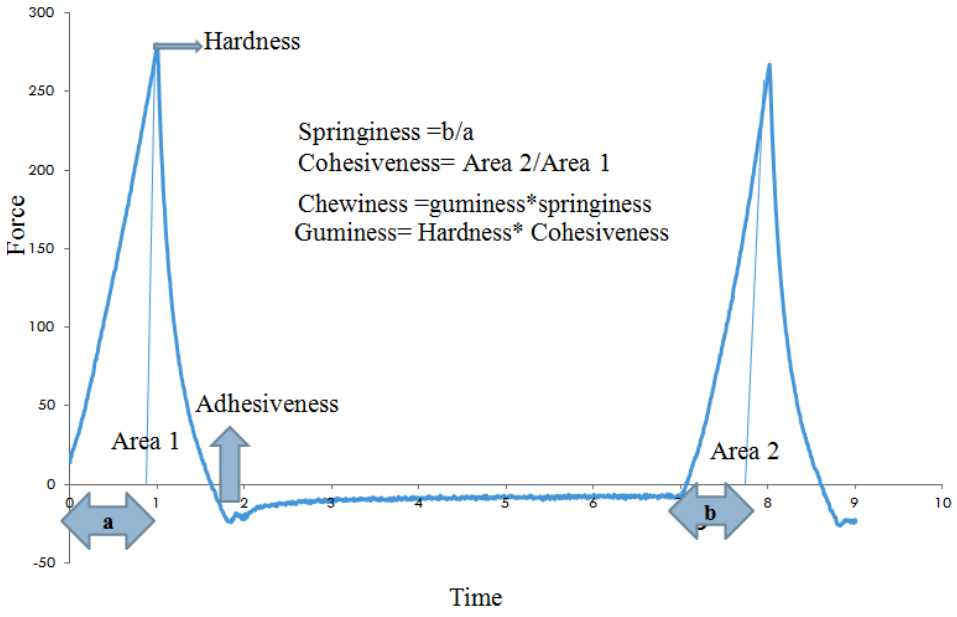


Figure 2.3 A typical Texture Profile Analysis (TPA) curve

2.5.5 Improvement of functional properties

HPP has shown promise to enhance these properties by processing without heat. Advantages of HPP include the ability to process food at lower temperature, without additives and foods with enhanced functional properties. There is always increasing demand for products that are naturally produced, fresh, retain their nutritional value and safe (Martin et al., 2002).

There is no damage to structure of most food products during HPP due to isostatic pressing. When food sample is immersed in the pressure- transmitting medium, external pressure on the food remains equal to the internal pressure. However, HPP has a significant effect on functionality and the rheological properties of a product. HPP effects molecular interactions resulting in affecting macromolecules such as proteins, carbohydrates, and lipids. Unlike thermal treatments, HPP does not affect covalent bonds such as cross linkages within macromolecules. The one exception is disulfide bonds in proteins. In starches, pressure generally raises the gelatinization temperature while increasing amylase digestibility. HPP ultimately destroys the granular structure of starches via hydration of the amorphous phase and distortion of the crystalline region. Similar to proteins, some carbohydrates also form gel with HP treatment (Heremans, 1995). HP treatment tends to increase peroxide values of lipids resulting from oxidation. Para-anisidine values are also increased, resulting in more secondary oxidation products (Ludikhuyze et al., 2000).

Eggs are very popular for their exceptional functional qualities. Egg white is known for foaming, leavening, gelling and binding agent in various food preparations. Egg proteins are heat sensitive and all functional properties (formability, foam density and viscosity) are affected by application of heat. The United States Department of Agriculture recommends the heating egg white and yolk to 57.5 and 61.1˚C respectively for 2.5 min to ensure safety/protection against salmonella. This level of heating can cause overheating and partial coagulation of proteins present in egg like ovalbumin, conalbumin, ovotransferrin, ovomucoid and ovomucin. HPP can be one of novel food processing technology which can cause effectively pasteurize without application of heat.

2.5.5.1 Gelation

The unfolding or denaturation is responsible for gelation in egg white which leads to formation of spherical aggregates due to hydrophobic interactions, resulting in more turbid solution. Aggregates start to thicken by stabilization via sulfhydryl-disulfide reactions which are followed by coagulation and gelation due to rapid formation of hydrogen bonds (Mine et al., 1995).

Ovotransferrin also plays an important role in gelation as it is the first egg white protein to thermally denature and initiate coagulation (Croguennec et al., 2002). Egg gels can be formed by either heat, HP treatment, or under acidic conditions. There are many factors like temperature, pressure, pH and salt concentration (ionic strength which affects the gel formation.). Various forms of egg gels can be formed due to thermal denaturation varying as per pH and ionic strength. When pH of egg is near its isoelectric point i.e. 4-5, the egg white proteins are highly denatured into protein aggregates randomly via hydrophobic interactions (Nakamura et al., 2000). As the pH reaches that isoelectric point, the net charge on the proteins is reduced resulting in an increase in hydrophobic interactions followed by aggregation. These conditions can result in opaque and turbid gels (Nakamura et al., 2000). It was found that protein gels heated above 80ºC are more prone to shrinkage and syneresis. On other hand, it was found that when pH is going further from isoelectric point; there is low ionic strength and the denaturation of proteins takes place leading to aggregation in an ordered and linear manner forming a transparent gel. Aggregates formed are smaller which forms a tighter gel network with increased water holding capacity (Barbut, 1998).

Both heat and HP treatments on Ovalbumin exposes the hidden –SH groups via the Ellman's reagent method (5, 5' - dithiobis - (2 - nitro benzoic acid)) (DTNB). These exposed groups tend to stabilize protein aggregates resulting in gelation. Hayashi et al., (1989) found that pressure induced gels are more glossy and smooth in appearance. On the other hand, gel formation can be prevented by using 10% egg white solutions prior to HP treatment (Lametti et al., 1999).

2.5.5.2 Foaming

The major egg white proteins that are important to foaming are ovalbumin, ovomucin, ovotransferrin and lysozyme (Mine et al., 1995). Ovalbumin plays a central role in egg white foaming abilities. Ovalbumin molecules are adsorbed in the air/water interface and the hydrophobic areas of the protein are oriented towards the gas phase of the interface during whipping. Protein may denature partially depending on extent of heat or pressure applied and resulting in overall affect on functional properties. Egg white

proteins comprise more than 80% of dry matter and they are mainly responsible for foaming and gelling. It was found that these functional properties were enhanced with the use of high pressure homogenization. It has been already reported that different proteins of egg white dominate different actions during foaming and gelation (Hsieh et al., 1993). The conformational changes in structure expose buried Sulfhydryl groups which then become oxidized. This results in the formation of disulfide bridges with adjacent Ovalbumin molecules. Aggregates are then formed at the air/water interface and produce a gel network that provides stability to the foam. Non covalent bonds and disulfide bridges are responsible for foam network which are formed due to denaturation and conformational changes. The other major factor affecting foam formation is the ability of protein to be adsorbed into air/water interface.

2.5.5.3 Protein digestibility

Digestion starts in the stomach by conversion of pepsinogen to pepsin by the action of hydrochloric acid, and the action continues by trypsin and chymotrypsin in the intestine. The gastrointestinal tract is responsible for absorption of amino acid and their derivatives (result of dietary protein). The absorption rates of amino acid vary according to the protein sources. Similarly, digestibility of protein depends upon their structure. Compact structure of protein prevents unfolding of the peptide chains which in turn decrease the digestibility by reducing accessibility of some peptide bonds to enzymes. Cross-linking can cause decrease in rate of protein hydrolysis due to heating, as well as by interactions of amino groups with reduced saccharides or other compounds containing carbonyl groups. In food processing, these are secondary products of lipid oxidation and aldehydes contained in smoked products. As a result of several further reactions, different unsaturated compounds are generated and they interact with the amines, possibly involving different cross-linking reactions (Matthews and Laster, 1965).

The most straightforward and scientifically sound approach for routine assessment of dietary protein quality for humans is called protein digestibility-corrected amino acid score (PDCAAS) method. It is a method of evaluating the protein quality based on both the amino acid requirements of humans and their ability to digest it. It was

adopted by the US Food and Drug Administration (FDA) and the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) in 1993 as "the preferred best" method to determine protein quality. These organizations have suggested that other methods for evaluating the quality of protein are inferior (FAO, Rome). Throughout food processing, protein sources are treated with heat, oxidizing agents such as hydrogen peroxide, organic solvents, alkalis, and acids for a variety of reasons such as to sterilize or pasteurize, to improve flavor, texture, and other functional properties, to deactivate antinutritional factors, and to prepare concentrated protein products. These processing treatments can result in maillard compounds, which in turn cause lowering of amino acid bioavailability and a decrease in protein quality (Bender, 1972).

Sarwar et al. (1990) have discussed the background information leading to the development of the protein digestibility-corrected amino acid score and its methodology. They included a brief review of information on rat growth assays for predicting protein quality, determination of digestibility of protein and bioavailability of amino acids in foods, and amino acid scoring procedures including reference patterns and amino acid methodology.

Similarly, a kinetic study was conducted on the effect of heating in the temperature range of 50−92°C, on the susceptibility of ovalbumin and albumen solutions to enzymatic hydrolysis by a mixture of trypsin and α-chymotrypsin at 37 °C and pH 8.0. Heat treatment caused increase in degree of hydrolysis after 10 min of enzymatic reaction of both ovalbumin and albumen. The time-dependent change in the susceptibility to enzymatic hydrolysis after heat treatment was described by a fractional conversion model. Different end levels of degree of hydrolysis were obtained after heating for a longer time at different temperatures, which suggested that the final degree of unfolding of the protein is temperature dependent (Plancken et al., 2003).

2.5.5.4 Protein water holding capacity (WHC)

WHC is an important property to determine the strength of the water binding to the solid matrix. It can be weaker in dehydrated and rehydrated foods than in original food. Pandey et al. (2000) studied the WHC of rennet curds obtained from HP-treated milk. They found that decrease in pressure level, temperature and holding time, caused a decrease in water-holding capacity and an increase in the gel strength of the produced rennet curds.

It was found that potato starch and egg white have great influence on water-holding capacity (WHC) and microstructure of surimi gels induced by high pressure (400 and 650 MPa, 10 min) as compared to heat-induced (90°C, 40 min) gels. Gels were treated by high pressure or heat, and then examined by scanning electron microscopy (SEM). Final moisture content was adjusted to 78 g/100 g and WHC was determined using a centrifuge method. SEM demonstrated that the surimi-egg white has a fibrous and homogeneous network structure, whereas surimi-starch exhibited a compact network. When potato starch was added alone or in combination with egg white, the integrity of some starch granules was maintained after pressure treatment, suggesting that high pressures at the 400 and 650 MPa levels do not affect the structure of potato starch. Additive treatment increased WHC of Pacific whiting surimi gels at 400 and 650 MPa with egg white, holding more water than heated gels (Munizaga et al., 2004).The amount of held water in a gel-like system would vary with all the factors that affect the equilibrium state of swelling of the gel, such as concentration of cross-links, solvent quality, pH and ionic strength (Foegeding, 2006).

2.5.6 Antioxidant activity

2.5.6.1 Overview

Oxidative reactions can lead to the deterioration of quality characteristics of food such as flavor, aroma, texture and color for example: Rancidity is one of oxidative reactions caused due to auto oxidation in which oxygen attacks at the lipid complexes leading to chemical changes causing production of off flavors in the food. The mechanism of oxidative rancidity of lipids has been well established (Bateman et al.,

1954). During rancidity, atmospheric oxygen reacts with organic compounds and degrades their structure, which is mainly responsible for the loss in quality of the chemical products of industrial importance. Oxidative stability of foods is dependent on balance between antioxidative and pro oxidative factors. The biological tissues from which foods are obtained contain numerous antioxidant systems to maintain the antioxidant/prooxidants balance in favor of antioxidative protection, thus helping in protecting these tissues from oxidative harm. The antioxidant system has free radical scavengers, metal chelators and enzymes that inactivate reactive oxygen species.

Food proteins are important components of foods and can also act as free radical scavengers, chelating agents for transition metals and decrease the radical damage in biological systems (Zielinski and Kozlowska, 2000). There are several studies that demonstrate the ability of proteins to inhibit lipid oxidation in foods. Proteins originating from milk, blood plasma, and soy protein all have been shown to exhibit antioxidant activity in muscle foods. Whey proteins have also been found to inhibit lipid oxidation in oil-in-water emulsions (Taylor and Richardson, 1980). Due to concern over the safety of synthetic compounds, special interest has been accrued on replacement of synthetic antioxidants by naturally occurring antioxidants. With growing trend of all natural products, natural food products with antioxidants will replace BHA and BHT. This interest has led to the new investigations into measuring the antioxidant potential of biologically active peptides from protein hydrolysates (Li et al., 2007). Egg Proteins also have ability to inhibit lipid oxidation. Therefore the oxidative stability of the foods can be increased by protecting the endogenous antioxidant enzymes, enhancing the activity of the proteins by altering the structure, and by using proteins and peptides with antioxidant activity as food additives.

It has been reported that peptides produced by enzymatic hydrolysis of crude egg white with pepsin have antioxidant activity. Four peptides included in the protein sequence of ovalbumin possessed radical scavenging activity higher than that of Trolox. The combined antioxidant and ACE inhibition properties make it a very useful multifunctional preparation for the control of cardiovascular diseases, particularly hypertension (Davalos et al., 2004). Egg yolk is also used as a functional and nutritional

ingredient in food products. Main components of egg yolk include triacylglycerols and phospholipids which are mainly used as food- or cosmetic-grade yolk-lecithin. Egg-yolk protein is produced as the residue of egg yolk (after extraction to give yolk-lecithin by organic solvent). Egg-yolk protein hydrolysates are prepared by the enzymic hydrolysis of the yolk protein, and the hydrolysates are water-soluble and have high nutritional value. The hydrolysis of food proteins causes formation of peptides which can act as antioxidants. Various foods such as egg yolk, soy, whey protein and muscle foods (beef, pork and tuna) can inhibit lipid oxidation (Sakanaka and Tachibana, 2006; Diaz and Decker, 2005; Sakanaka et al., 2005).

Similarly antioxidant properties of the barley glutelin hydrolysates were evaluated based on their radical scavenging capacity (DPPH method). Alcalase hydrolysates (AH) demonstrated significantly higher antioxidant capacity than those treated by flavourzyme in most of the assays. Hydrolyzed barley glutelin is a potential source of antioxidant peptides showing importance of hydrolysis on antioxidant activity (Xia et al., 2012).

2.5.6.2 Mechanism of autoxidation

Protein can inhibit oxidation by biologically designed mechanism. These types of antioxidative proteins contribute to the endogenous antioxidant additives. The antioxidant activity of proteins is due to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, enzymatically eliminate specific oxidants, and alter the physical properties of food systems in a way that separates reactive species. Proteins can potentially act as multifunctional antioxidants that can inhibit several different lipid oxidation pathways. Free-radical chain reaction is the direct reaction of a lipid molecule with a molecule of oxygen (Jadhav et al., 1996). Autoxidation follows following mechanism:

1. Chain initiation: The chain initiation occurs with the formation of free radicals.

$$RH \rightarrow R^{\circ} + H^{\circ}$$

$$R^{\circ} + O_2 \rightarrow ROO^{\circ}$$

The formation of lipid free radical R° mainly occurs due to light or heat, trace metals, irradiation and alkaline conditions. After oxidation reaction, lipid hydroperoxides break down to yield radicals.

$$ROOH \rightarrow RO^{\circ} + HO^{\circ}, \text{ and}$$

$$2ROOH \rightarrow RO^{\circ} + ROO^{\circ} + H_2O$$

2. **Chain propagation**: In chain propagation reaction, the lipid radicals are converted into peroxy radicals by reacting with molecular oxygen.

$$R^{\circ} + O_2 \rightarrow ROO^{\circ}$$

$$ROO^{\circ} + RH \rightarrow ROOH + R^{\circ}$$

Chain initiation reaction occurs due to the presence of lipid peroxy radicals (ROO°) and it results into the formation of lipid hydroperoxides and lipid free radicals. Chain propagation reaction continues as long as unsaturated lipid molecules are available.

3. **Chain termination** occurs when there is a reduction in the amount of unsaturated lipid molecules present, radicals bond to one another, forming a stable non-radical compound. Chain propagation stage can be prevented by the presence of natural antioxidants, which break the chain reaction (Coulter, 1988). Vitamin E is a major lipid soluble chain breaking antioxidant and prevents lipid oxidation by scavenging or converting free radicals into less reactive forms.

$$TH + X^{\circ} \rightarrow T^{\circ} + XH$$

X° = Free radical, TH = Tocopherol, T° = Tocopheroxyl radical

Tocopheroxyl radical is further converted into tocopherol by using water soluble antioxidants such as ascorbate (Chan et al., 1994; Mukai et al., 1990).

Butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT), and propyl gallate are synthetic antioxidants. Their use is limited for BHA, BHT (100-200 ppm) or TBHQ (200-500 ppm) and propyl gallate for the stabilization of fats and oils. The increasing demand for “all natural” products is due to limitations on the use of synthetic antioxidants and enhanced public awareness of health issues. Natural antioxidants are “generally recognized as safe” (GRAS) to be used in food

components. Many natural food components like oils and oilseeds, proteins and protein hydrolysates, fruits and vegetables, oat and rice bran, spices, herbs and tea have antioxidant properties. Natural antioxidants from these food components provide oxidative stability to the food product.

As antioxidative properties of proteins contribute to the endogenous antioxidant capacity of foods, therefore proteins can also be used as potential antioxidant additives.

2.5.6.3 Increasing the antioxidant activity (AA) of proteins

Increasing the exposure of antioxidant amino acids in proteins can also be accomplished by enzymatic hydrolysis. Increased antioxidant activity in hydrolyzed proteins has been reported for milk (Ostdal et al., 1999). The observed increase in antioxidant activity due to hydrolysis may result from increased solvent exposure of amino acid which could lead to increased metal chelation capacity (Wang and Xiong, 2005). The main reason for antioxidant activity is their physical properties and chemical composition.

The antioxidant activity of proteins can also be increased by interactions with reducing sugars to produce Maillard reaction products (Zamora and Hidalgo, 2005). Protein-reducing sugar Maillard products include a broad array of structures that contain furans, reductones, Schiff bases, and aldehydes. The Maillard reaction products can inhibit lipid oxidation by free radical scavenging and metal chelation. Augustin et al. (2006) found that by heating casein stabilized fish oil-in-water emulsions in the presence of glucose resulted in the formation of Maillard reaction products that were capable of inhibiting lipid oxidation.

2.5.6.4 Methods of determining antioxidant activity (AA)

There are various methods of determining antioxidant activity of protein sources. These methods determine antioxidant activity by judging the ability of proteins to scavenge free radicals like 1, 1-diphenyl-2-picrylhydrazyl (DPPH).

a. DPPH method: The scavenging effect of proteins and protein hydrolysates fractions on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical is measured according to the

method of Church et al., 1992 with little modification. A protein solution is prepared and after centrifugation at 8000 RPM for 20 min, 2 mL of the supernatant is added to the 2 mL solution of 0.1 mM DPPH dissolved in 95 % ethanol. This solution is then mixed properly and left for 30 min in dark at room temperature. The absorbance of resulting solution is read at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect is mainly expressed as shown in the following equation:

$$DPPH\ scavenging\ activity\ (\%) = \left[\frac{(Control\ absorbance - sample\ absorbance)}{Control\ absorbance}\right] \times 100 \qquad (2.3)$$

Control consists of 2 mL of ethanol and 2 mL of 0.1 mM DPPH.

b. Superoxide Radical Scavenging assay (SRS Assay)

SRS assay of proteins is measured using spectrophotometric method of inhibition of pyrogallol autoxidation as described by Marklund and Marklund (1974).This assay is dependent on the reducing activity of a test compound by O_2 dependent reaction, which releases chromophoric products. In this method, 0.1 mL of the protein solution is added into 2.8 mL Tris-Hcl-EDTA buffer (0.1 M, pH 8.0), and the mixture is shaken and heated at 25° C for 10 min. After 10 min of heating, the reaction is initiated by adding 0.1 mL of pyrogallol solution (3 mM) and then optical density is measured at 325 nm using a spectrophotometer. The scavenging activity is given as following:

$$Scavenging\ activity(\%) = \left[\frac{(CA - SA)}{CA}\right] \times 100 \qquad (2.4)$$

where CA = Control absorbance, SA = Sample absorbance

c. Test of inhibition of linoleic acid autoxidation by proteins

The antioxidant activity of proteins and protein hydrolysates with different periods of incubation in a linoleic acid model system is measured by the method of Osawa and Namiki (1985). 10 mg of protein or protein hydrolysate is dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and then the mixture is added to a solution of 0.15 mL linoleic acid and 10 mL 99.5 % ethanol. Total volume is then adjusted to 25 mL with

double distilled water. The mixture is then incubated in a conical flask at 40°C in a dark room for 6 or 8 days, and degree of oxidation is mainly evaluated by measuring the ferric thiocyanate (FTC) values.

FTC value is mainly measured according to the method of Mitsuta et al. (1996). According to this method, 100 µl of the above reaction is mixed with 4.7 mL of 75 % ethanol, 0.1 mL 30 % ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride solution in 3.5 % HCL. After the reaction time of 3 min, the FTC value is measured by reading the absorbance at 500 nm to know the inhibition of linoleic acid autooxidation.

d. Hydroxyl radical-scavenging activity

The effect of hydroxyl radicals can be assayed using the 2-deoxyribose oxidation method (Chung et al., 1997). 2-Deoxyribose is oxidized by hydroxyl radicals formed by the Fenton reaction and degrades to malondialdehyde (Gutteridge, 1984; Halliwell, 1997). The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$–EDTA, 0.15 mL of 10 mM hydrogen peroxide, 0.525 mL of distilled water, and 0.075 mL of the sample solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37 ℃ for 4 h, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice, and then measured at 520 nm. Hydroxyl radical-scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radicals. The results were calculated as the percentage inhibition according to the following formula:

$$\%inhibition = \left[\frac{\{(C-CB)-(S-SB)\}}{(C-CB)}\right] \times 100 \qquad (2.5)$$

where S, SB, C, and CB are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

e. Antioxidant activity against lipid peroxidation

This method involves homogenizing ground beef and tuna (20% w/v and 10% w/v) in 50 mM HEPES buffer for 5 min using a homogenizer. 0.8 mL of homogenate (beef or tuna) and 0.2 mL of HEPES buffer solution was incubated at 37 °C for 60 min. After incubation, the mixture was tested for the formation of thiobarbituric acid reactive-substances (TBARS). TBARS were determined by method used by Lee and Hendricks, 1997 with little modification. Trichloroacetic acid (TCA/TBA) stock solution was prepared consisting of 15% TCA (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. After mild heating and agitation to dissolve the components, 3 mL of 2% butylated hydroxytoluene (BHT) in absolute ethanol was added per 100 mL of the TCA/TBA stock solution. At appropriate intervals, 1.0 mL of aliquot of the sample medium was added to the TCA/TBA stock solution in a test tube and immediately mixed thoroughly with a Vortex mixer. The sample was first heated in a boiling water bath for 10 min and cooled to room temperature, following by centrigugation at 1710 x g for 10 min. The absorbance of the supernatant was measured at 532 nm using a UNIDEC-50 spectrophotometer. TBARS were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxypropane (TEP).

2.6 **Response surface methodology**

Response surface methodology (RSM) is an empirical modeling technique used to estimate the relationship between a set of controllable experimental factors and observed results.This method was first introduced by Box and Wilson in 1951. The main idea behind using response surface is to use sequence of designed experiments to develop, improve and optimize a process. In addition, it has important applications in the design, development and formulation of new products and in the improvement of existing product designs. RSM is widely used in academic and industrial units where independent variables influence the quality characteristics of response variables of the product or process. These designs provide information on direct effects, pair wise interaction effects and curvilinear variable effects. RSM is one of the approaches to product and process

optimization work. Central composite rotatable design (CCRD) is most popular experimental design used in response surface methodology due to following factors:

1. These are very efficient providing information on experiment variable effects and overall experimental error in minimum number of runs required.
2. These are very flexible as they can work under different experimental regions of interest and operability.
3. These can run in sequential manner as it can be partitioned into two subsets, first can be used to estimate linear and interaction effects while the second can be used to estimate curvature effects.

2.7 Practical challenges in high pressure processing

Most of High Pressure applications in food are not only pressure dependent but also temperature dependent. The main system factors affecting high pressure are temperature, holding time, mode of pressurisation, pressure level and pressure non-uniformity.

2.7.1 Pressure level

The level of pressure applied is an important factor affecting quality of pressure treated product. A direct relationship between magnitude of applied pressure and resulting effects are observed. Effects of pressure on most of biochemical processes are, reversible at low pressure (100-200 MPa) whereas non reversible effects occur above 300 MPa (Gross and Jaenicke, 1987). Lower pressure may be more beneficial for destruction of microbial spores than directlyusing higher level of pressure because low pressure may induce germination of spores. It was seen that spores of *Alicyclobacillus acidoterrestris* and *Bacillus coagulans* can be germinated using high pressure level of 100-300 MPa and this part followed by HP treatment of upto 800 MPa showed highest inactivation at 60°C (Vercammen et al., 2012).

2.7.2 Pressurization mode

High pressure may be applied either in static (pressure hold) or dynamic (pressure pulse) mode. Static mode means increasing pressure to certain level followed by holding for specified length of time before pressurization. Main advantage is that once the certain required pressure level has been achieved, holding does not require extra input energy. Level of pressure applied and holding time are directly related to mode of pressurization. But it is cost effective to process at higher pressure as processing time and number of processing cycles would be reduced. Pulse pressurization is technique where successive series of pressurization and depressurization applications are given at desired pressure level without any holding time. Pulse pressure causes bursting of cell walls that kill the micro organisms (Zobell, 1970). Pulse pressurization of bacteria in a system comprising of compressed much more effective compared to high pressure forces gas into solution that diffuses into bacterial cells and sudden depressurization may cause expansion of gas.

2.7.3 Heat transfer rate during pressurization

The physical compression causes increase in temperature during high pressure processing. The increase in temperature is dependent on number of factors including initial temperature, material compressibility, material composition, specific heat and target pressure. The rate of temperature increase of the water like substances is in phase with changes in pressure, food with fatty substances often exhibit a time delay of 30–60 seconds before reaching the maximum temperature. The main possible reason is difference in their molecular structure (Rasanayagam et al., 2003). During the actual process, in the holding time phase, the temperature of the product decreases due to heat loss through the pressure level. The product temperature returns to a little lower than the initial temperature. Thus, high pressure offers a unique way to increase the temperature of the product only during the treatment.

2.7.4 Process modeling

The temperature of a homogeneous food will increase uniformly due to compression, but variation in temperature distribution can develop during the holding

period due to heat transfer between system (food) and surroundings (pressurization medium and pressure vessel). The temperature of the pressurized food will be greater than the pressurising medium during pressurization due to compression heating. This phenomenon will lead to temperature gradient between pressurized food and pressurizing medium (Rasanayagam et al., 2003). This difference can result in variation in enzymatic/microbial inactivation levels, as well as nutritional quality degradation (Denys et al., 2000b); they have also adopted a numerical approach for modelling conductive heat transfer during HPP and demonstrated that the non uniform temperature distribution can result in non-uniform enzymatic inactivation.

It is clear from the above literature search; HP processing can be used for many food products and for several different purposes. The present thesis focuses on using HPP for enhancing several functional properties of egg products.

PREFACE

TO CHAPTER 3

From the literature review, it is clear that high pressure processing (HPP) can be used to improve structural and functional properties of foods and also reduce the anti-nutritional properties of egg components. In this Chapter, the effect of HPP on destruction kinetics of avidin (anti-nutritive factor) will be elaborated. Avidin is an anti nutritive factor which causes the binding of biotin (vitamin B_7). Prolonged deficiency of biotin can cause number of health problem related to human skin and thyroid. Even for process establishment of HPP for egg components, this information can prove very useful as HPP had advantage in terms of reducing activity over conventional thermal processing.

Part of this research was presented in 2009 at the Non thermal processing division workshop and conference, Montreal, Canada. One manuscript has been prepared from this Chapter.

Singh A and Ramaswamy HS (2012) Comparative study of combined high pressure and thermal treatment versus conventional thermal processing: An analysis of avidin inactivation kinetics (Prepared for Submission)

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy.

CHAPTER 3
EVALUATION OF HIGH PRESSURE INACTIVATION KINETICS OF AVIDIN IN EGG PRODUCTS

3.1Abstract

High pressure inactivation kinetics of the enzyme avidin was evaluated at different pressure and temperature combinations and compared with conventional thermal inactivation kinetics. Both pure avidin and avidin from raw egg were studied for their inactivation with pressure. The treatment were given at 500-700 MPa and 80-100°C for pure avidin and at 500 to 700 MPa and 30 to 50°C for avidin present in egg white (EW) and whole liquid egg (WLE). Avidin activity was found to be sensitive to pressure and was well described by the log-linear first order model with D and z value concept. For pure avidin, the highest severity combination of 100°C and 700 MPa gave the least D value of 26 min. On the other hand, the conventional thermal treatment at 100°C gave a very high D of 111 min, almost 5 times higher. Pure avidin added to EW and WLE gave D values of 10.9 and 10 min for EW and WLE, respectively indicating the increased sensitivity of the enzyme to pressure inactivation in the raw egg medium. The avidin present naturally in egg was even more sensitive to pressure inactivation with D values at 50°C and 700 MPa of 2.2 and 2.2 min for avidin in egg white (EW) and whole liquid egg (WLE), respectively. They were completely inactivated at the higher pressure and temperature levels employed for pure avidin. The pressure and temperature sensitivity parameters (z value) were also determined both as a function of pressure at different temperatures and as a function of temperature at different pressure levels.

3.2 Introduction

Egg is a complete nutritional food in itself, providing an inexpensive and a low-calorie source of high-quality protein. Apart from this, it possesses functional characteristics such as antimicrobial, mineral binding, growth stimulating and

antioxidant properties as well (Korhonen et al., 2006; Murray et al., 2007). Egg protein mainly contains ovalbumin, ovotransferrin, ovomucid, ovoglobulin, ovomucin, lyzozyme and avidin. Egg white protein like ovalbumin is a multifunctional component which is used in variety of food preparations for its gelling, foaming and emulsifying characteristics. Correlation of structural and functional properties of egg white is a major focus of studies employing egg proteins. On the other hand, egg protein like avidin (tetrameric structure: 66-69kDa), possesses some anti-nutritional properties. It can bind to biotin (Vitamin B_7) and makes it inaccessible to the body. Biotin is an important vitamin, necessary for cell growth and metabolism of fats and amino acids in the human body (Laitinen et al., 2007). It has been shown that one mole of avidin has the ability to bind four moles of biotin (Korpela, 1984). Avidin is highly stable to processing treatments, therefore thermal and non thermal processes aim at breaking this non covalent bonding, so as to make biotin available for digestion and absorption by the body.

Thermal processing is the most commonly adopted method for egg preservation, especially ensuring microbial inactivation, but protein avidin is highly resistant to this treatment (Durance and Wong, 1992). Several studies have been carried out on inactivation of avidin-biotin complex by high temperature treatment (Green, 1962; Green 1966; Donovan and Ross, 1973; Durance and Wong, 1992). Apart from high resistance of avidin to heat, a major drawback in these studies performed was found to be extensive changes in egg quality in terms of their emulsifying and binding properties.

Non-thermal method is a prospective alternative to inactivate avidin and enhance nutritional value of egg and egg products. High pressure processing (HPP) is a novel non thermal food processing method which can be used to break strong non covalent bonds (Mozhaev et al., 1996). In addition, HPP can cause inactivation of microorganisms, denaturation of proteins and shelf life extension, that helps in preserving quality of food in better way as it gives fresh like appeal in food (Ahmed et al., 2003). The effect of HPP on proteins has already been studied for several decades but it gained significant interest when potential applications and limitations of food proteins were published in late 1990's (Knorr, 1990). These applications of HPP have lead to development of food products with added functional and health benefits (Balasubramaniam et al., 2008).

HPP can also cause modifications in secondary and higher order structures of protein at pressure level more than 200 MPa through disruption of ionic and hydrophobic interactions (Messens et al., 1997; Huppertz et al., 2002). Ahmed et al. (2003) revealed that HPP causes coagulation of WLE (whole liquid egg) and EW (egg white) at pressures higher than 300 MPa. Therefore, HPP can be used to induce desired denaturation of proteins, and inactivation of enzymes. As all enzymes are proteins, pressure inactivation of enzymes depends on the type and source of enzyme; nature of the medium in which enzyme is dispersed, pressure, temperature and treatment time (Knorr, 1966; Kunugi,1992; Hendrickx et al., 1998; Hayashi et al., 1998). The ability of the HPP to inactivate enzymes and destroy microorganisms has encouraged the food industry to introduce HPP foods to the market (Hayashi, 1989; Alvarez et al., 2008).

The present study is focused on destruction of avidin-biotin complex using high pressure in combination with high temperature. Varying levels of pressure-temperature treatments were employed to study the avidin destruction kinetics. This study is novel in its kind, as there is no reported study for avidin inactivation, using high pressure processing. The main objective was to execute comparative evaluation of high pressure in combination with high temperature (HP+HT) versus traditional thermal treatment of the biotin binding activity of avidin in pure avidin (egg source) and raw egg components. This study will be a gateway to understand and improve nutritional value of egg and egg products using HPP.

3.3 Materials and methods

3.3.1 Chemicals used: 2-(4'-hydoxyazobenzene) benzoic acid (HABA), D-biotin and avidin from egg source (Purity ≥98% SDS-PAGE Grade) were bought from Invitrogen corporation (Burlington, Ontario).

3.3.2 Sample preparation

3.3.2.1 Preparation of pure avidin solution: 0.6 mg/mL avidin solution was prepared using 0.02 M sodium phosphate buffer (pH 7.3). Avidin solution was sealed into capillary tubes. Samples were equilibrated to 20°C before processing treatments.

3.3.2.2 Preparation of raw egg components

Fresh raw large A size eggs were procured from a local grocery store. All inferior quality eggs were removed. Eggs were washed properly to remove any traces of dirt if present. The eggs were manually broken and yolks were clearly separated from the EW. The eggs were broken down and blended properly for whole liquid egg. The liquid egg samples were mixed properly and packed in ampoules (4 ml whirl pak ampoules,WhirlPak$^{(R)}$, Canada) for processing treatments. Avidin analysis was done after high pressure treatments. Denaturation had no effect on avidin activity analysis as samples were centrifuged to remove any denatured particles.

3.3.3 Thermal treatment

3.3.3.1 Thermal treatment equipment: A glycerin bath (SL26, Julabo, Labortechnik GMBH, Germany) was used to treat samples at 80, 90 and 100°C.

3.3.3.2 Thermal treatment of pure avidin: These were given for pure avidin samples in buffer in pre stabilized glycerin bath at selected temperatures (80, 90, 100°C) with treatment times varying from 0-45 min (Table 3.1). The come up time ranged from 15-20 seconds depending on the desired temperature. The avidin destruction during come up time was found to be very small hence the effects of come up time period were neglected. This elimination did not restrict the scope of kinetic studies. After treatment, samples were taken out and kept in ice bath so as to prevent avidin inactivation during post process period. Samples remained submerged in ice bath until further evaluation

3.3.3.3 Thermal treatment of raw egg components: These time-temperature treatments were given for avidin naturally present in egg using a pre stabilized water bath at selected temperatures between 80 and 100°C for selected time intervals (0, 2, 4, 6, 8, 10 min). Full experimental details are given in Table 3.1. The come up times in these samples to reach the target temperature in test samples ranged from 90-120 seconds depending on desired processing temperature. Higher come up time was a result of the increased viscosity of egg samples providing resistance to heat penetration. Samples were treated for five different time intervals ranging from 0-10 min. After treatment,

samples were immediately submerged in ice bath to prevent further avidin inactivation. Time correction studies were performed so as to correct kinetic values.

Table 3.1: Holding times and temperatures used for thermal treatment

Product	Time(min)	Temperature(°C)
Pure avidin	0,15,30,45	80,90,100
Raw egg	0, 2, 4, 6, 8, 10	80,90,100

3.3.4 High pressure equipment and treatment

3.3.4.1 High pressure equipment: The hydrostatic pressure vessel used for this study was a batch type Unipress High pressure processing unit (U111 apparatus, Warsaw, Poland) equipped with a Huber thermal bath. This system can operate at pressures up to 700 MPa and at temperatures varying from - 40 to 100°C. The pressure come up times varied from 40 to 60 sec depending on the selected pressure level as higher pressure level required more come up time. The depressurization time was less than 25 seconds.

3.3.4.2 High pressure treatments for pure avidin: Wheaton ampoules containing samples were used in pressure processing chamber containing silicon oil (pressurizing medium). Various combinations of pressure (500, 600 and 700 MPa) and process temperature (80, 90 and 100°C) were used and duplicated samples were assessed for each treatment combination (Table 3.2). These particular pressure levels and holding times were used by taking into account the capacity of the HPP unit and the expediency of the kinetic assessment. Pressurization and depressurization rate of 4.4 MPa/s and 26 MPa/s respectively, were used during high pressure treatments

3.3.4.3 HP treatments for raw egg components: For raw egg components, HP treatments were given at pressure ranging from 500-700 MPa in combination with temperature from 30-50°C (process temperature) at different time intervals ranging from 2-10 min in order to monitor the destruction kinetics of avidin in EW and WLE (Table 3.2). Low temperature (30-50°C) and low time conditions were used for raw egg components (EW and WLE) because avidin was fully destructed at higher temperatures; there was no visible avidin activity in egg components. The increase in medium and

sample temperature during the treatments was found to be insignificant as pressurization was done at slow rate and heat produced during pressurization was dissipated from chamber at same time eventually preventing any significant rise in temperature of sample. T-type thermocouple attached to a temperature data logger was used for monitoring temperature changes during the process treatment. All treatments were carried out in duplicates and results were averaged for each sample. After treatment, samples were taken out and kept in ice water bath so as to prevent any avidin inactivation during post treatment period.

Table 3.2: Pressure levels, holding times and process temperatures used for HPHT treatment of pure avidin and raw egg

Product	Time (min)	Temperature**(°C)	Pressure*(MPa)
Pure avidin	0	80, 90, 100	500, 600, 700
	15	80, 90, 100	500, 600, 700
	30	80, 90, 100	500, 600, 700
	45	80, 90, 100	500, 600, 700
	Pressure*(MPa)	**Temperature**(°C)**	**Time (min)**
Raw egg	500	30, 40, 50	0,2,4,6,8,10
	600	30, 40, 50	0,2,4,6,8,10
	700	30, 40, 50	0,2,4,6,8,10

* Pressure SD was within 2.0 MPa for holding mode and maximum pressure.

**The deviation of initial temperature was within 1°C for all experiments.

3.3.5 Avidin extraction from raw egg components: Avidin was extracted from raw eggs following modified Durance method (1991). Processed egg (high pressure and temperature) and 0.2 M sodium phosphate buffer (pH 7.9) containing 0.25 M NaCl were taken in equal amounts. They were homogenized for 20 sec in a centrifuge tube by using Power Gen 700 (Fischer Scientific) homogenizer. Sample pH was adjusted below 7.9, whenever necessary. Homogenization gives milky suspension of coagulated egg sample with sodium phosphate. Milky suspensions were centrifuged by using Multi RF

Centrifuge (thermo electron corporation, MA, USA) at 15000 × g for 30 min and clear supernatant was used for avidin assay analysis. The specific gravity was assumed to be 1.0 for purpose of calculations.

3.3.6 Avidin assay: The avidin activity was assayed in duplicates from each container based on technique followed by Green (1965).

3.3.7 Kinetic data analysis: Combined pressure and temperature inactivation kinetics of avidin were studied at constant temperature during holding time and it was found to follow first order inactivation kinetics**.** The slope of linear regression of log avidin activity versus time at combined pressure and temperature treatments was used to calculate D at that temperature by using the following equation.

$$log\frac{A}{A_o} = -kt \tag{3.1}$$

A is residual avidin activity after pressure treatment of time t (min); A_o is initial enzyme activity; k is reaction rate constant, it was obtained from linear regression of log A/A_o versus time as negative slope:

$$k = \frac{-1}{slope} \tag{3.2}$$

D is referred as decimal reduction time or time (min) required at given pressure to cause 10 fold decrease in existing enzyme activity (i.e. resulting in 90% inactivation of initial activity at given pressure and temperature combination). In accordance with thermal processing theory, the temperature inactivation kinetics of microorganisms at different constant pressures was analyzed by the thermal death time (TDT) model. In this model, the thermal resistance of the D values at a constant pressure is determined by plotting the logarithm of D value against the temperature (T). From the regression of 1og (D) vs. T, the thermal resistance (Z_T) was evaluated as the negative reciprocal of the slope as shown below:

$$z_T = \frac{(T_2 - T_1)}{\log\left(\frac{D_{T1,P}}{D_{T2,P}}\right)} = \frac{-1}{slope} \tag{3.3}$$

D_1 and D_2 are D values at temperature T_1 and T_2 respectively.

Pressure resistance was evaluated by pressure death time model (PDT). In this, pressure resistance at constant temperature was determined by plotting log of decimal reduction time (D) v/s pressure. From regression of log D v/s pressure, the pressure resistance z_P value was determined as negative reciprocal of slope:

$$z_P = \frac{(P_2 - P_1)}{\log\left(\frac{D_{P1,T}}{D_{P2,T}}\right)} = \frac{-1}{slope} \quad (3.4)$$

D_1 and D_2 are D values at pressure P_1 and P_2 respectively.

Z_P of process is defined as pressure range between which the decimal reduction time is changed by 10 fold. Time correction studies were performed to examine and improve the accuracy of experimental data. It was used to correct kinetic values and account for small temperature variations during process treatments (Figure 3.1).

3.4 Results and discussions

Avidin activity was studied under different pressure and temperature regimes and its inactivation profile was analyzed. It was observed that the combination of pressure and temperature was more efficient in inactivating avidin enzyme than temperature treatments alone because pressure was acting in synergistic way with temperature.

3.4.1 Effect of thermal treatment

3.4.1.1 Effect of high temperature on avidin inactivation in pure avidin

Avidin activity inactivation in pure avidin as function of temperature and time is presented in Figure 3.2. Log of residual avidin activity v/s time after thermal treatment was plotted and it clearly indicates that higher temperature and time treatment resulted in higher rate of avidin inactivation. By increasing temperature (80, 90 and 100°C), D value was found to be decreasing (333, 169 and 132 min) (Table 3.3).

Avidin inactivation data for 80, 90 and 100°C was used for z value regression which was found to be 49.5°C. This study was similar to that reported by Wei and Wright (1964) i.e. $D_{100°C}$ = 123 min. Durance and Wong (1992) also reported that $D_{100°C}$ for pure avidin activity inactivation was found to be 107 min.

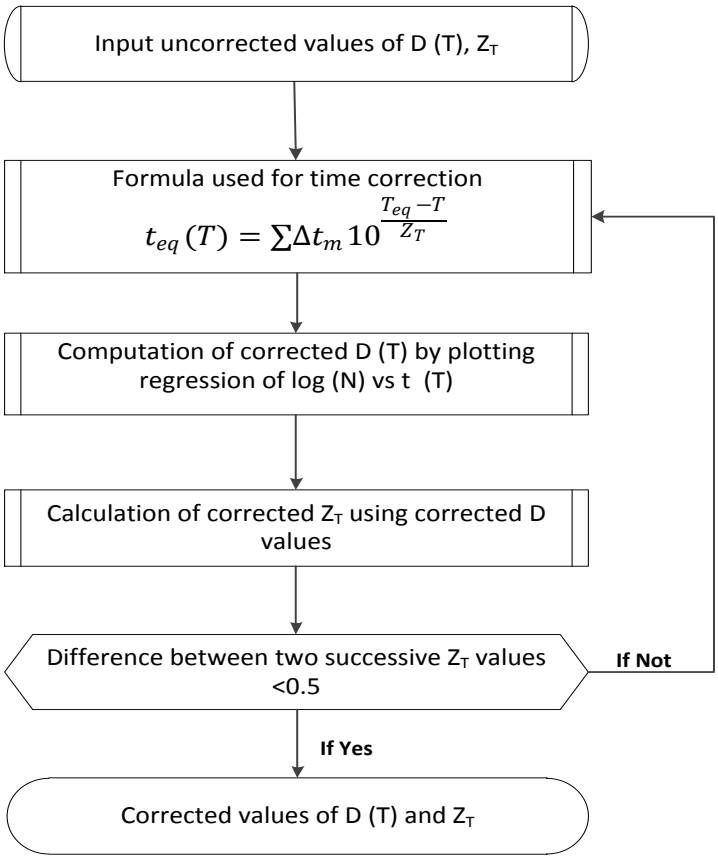


Figure 3.1: Schematic plan for time correction in thermal treatments

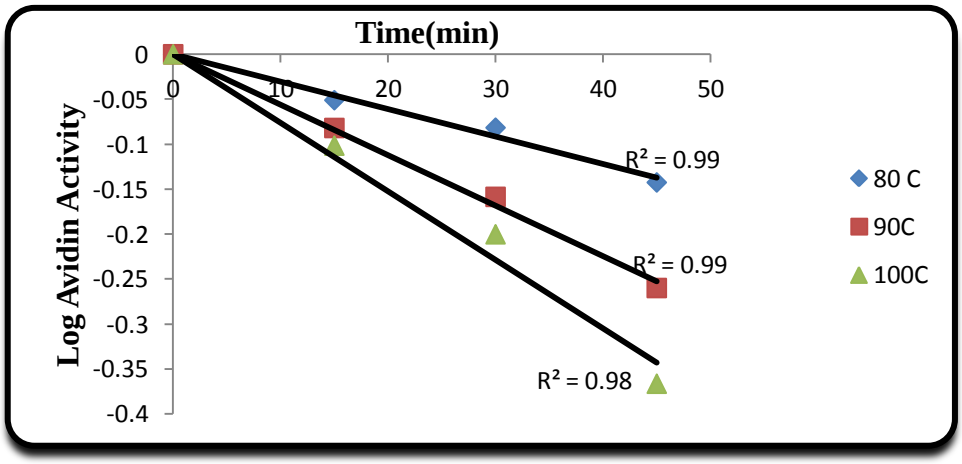


Figure 3.2: Effect of thermal treatments on pure avidin inactivation

3.4.1.2 Effect of high temperature on avidin inactivation in raw egg components

Reduction of avidin activity in egg white was observed with increase in temperature from 80-100°C. Corrected D values were found to be 9.38, 4.63 and 3.24 min at 80, 90 and 100°C in egg white respectively (Table 3.4). Lower z-values indicate higher pressure sensitivity of inactivation rates. On plotting log D values versus temperature, corrected z - value of 43.3°C was obtained (Figure 3.3). Similarly, in case of whole liquid egg, a corrected D value of 8.73, 5.33 and 3.35 min was found at 80, 90 and 100°C respectively (Table 3.4).

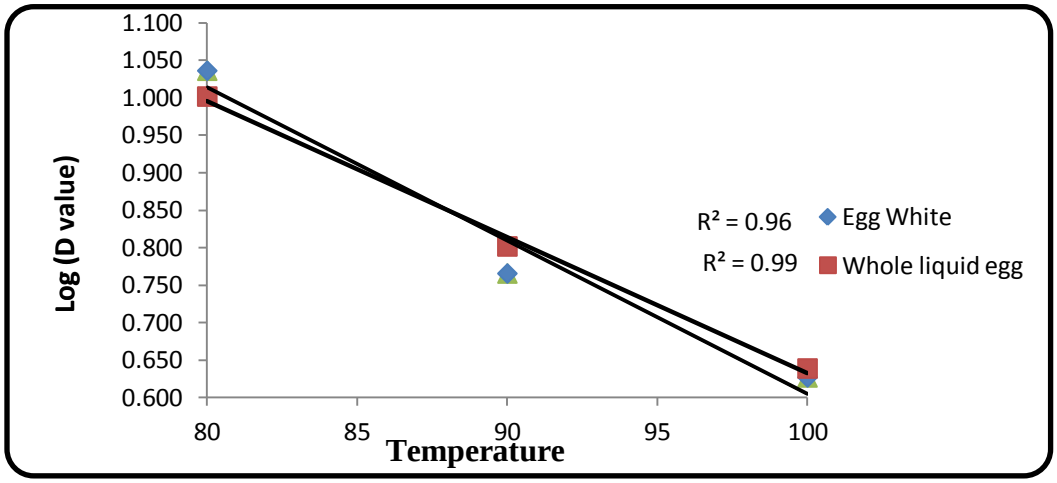
Table 3.3: Avidin inactivation caused by thermal destruction in pure avidin

Temperature	D value(min)	R^2	Z value	R^2
80	333	0.92		
90	169	0.99	49.5	0.94
100	132	0.95		

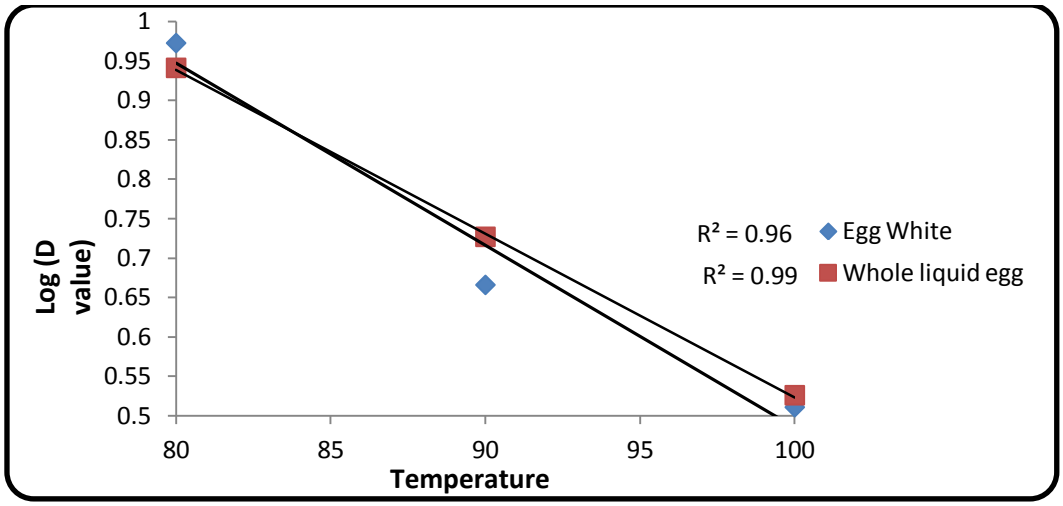
Table 3.4: Decimal reduction time of avidin activity in egg white and whole liquid egg subjected to thermal treatment

Egg White	**Uncorrected D Value**	**R^2**	**Corrected D value**	**R^2**
80	10.9	0.988	9.38	0.992
90	5.83	0.918	4.63	0.981
100	4.23	0.925	3.24	0.98
Whole liquid egg	**Uncorrected D Value**	**R^2**	**Corrected D value**	**R^2**
80	10.0	0.996	8.73	0.974
90	6.33	0.968	5.33	0.993
100	4.35	0.918	3.35	0.978

Corrected z - value was found to be 48.1°C for whole liquid egg (Table 3.5). Table 3.5 presents uncorrected and corrected z- value of avidin activity in egg white and whole liquid egg after thermal treatments for various time intervals. Data shows linear behavior in semi log plot. Durance (1991) found that mean residual avidin activity in fried, poached and boiled (2 min) egg white was 33.71 and 40% respectively of the activity in raw egg white.



(a)



(b)

Figure 3.3: a) Uncorrected and b) Corrected z value of avidin activity in egg white and whole liquid egg subjected to different thermal treatments at atm. pressure

3.4.2 Effect of high pressure (HP) on avidin inactivation

3.4.2.1 HPHT enzyme inactivation of pure avidin

To study the effect of pressure and temperature on inactivation of avidin, experiments were performed using high pressure (500, 600 and 700 MPa) in combination with temperatures of 80, 90 and100°C.

Results from pressure treatments at each temperature are shown in Figures 3.4, 3.5 and 3.6, respectively. The pressurization and depressurization without any holding time (pressure pulse) had minimal effects at pressures ranging from 500-700 MPa. From log residual avidin activity versus time plot, it was clearly visible that avidin activity showed diminution with increment in treatment intensity (Figures 3.4-3.6). The avidin inactivation was enhanced by high pressure application following semi logarithmic trend with treatment time (first order reaction).

Table 3.5: Corrected and uncorrected z value of avidin activity subjected to thermal treatments

Temperature	D value(min)	R^2	Z value	R^2
80	333	0.92		
90	169	0.99	49.5	0.94
100	132	0.95		

The D value was found to be 322 min at 500 MPa/80 °C which was reduced to 33.9 min at 700 MPa/100 °C (Table 3.6). Coefficient of determination (R^2) was found to be 0.95 or greater in most of the cases. In this study, avidin inactivation kinetics showed consistency with the statement that avidin is highly stable to thermal treatment but less stable to high pressure when used in combination with high temperature.

When high-pressure high-temperature (HPHT) processing treatments were compared with thermal treatments at the same temperature conditions (80, 90 and 100 °C), it was recognized that HPHT process at 100°C with 700 MPa resulted in approximately 5 fold decrease in D_{100^oC} values as compared to thermal processing.

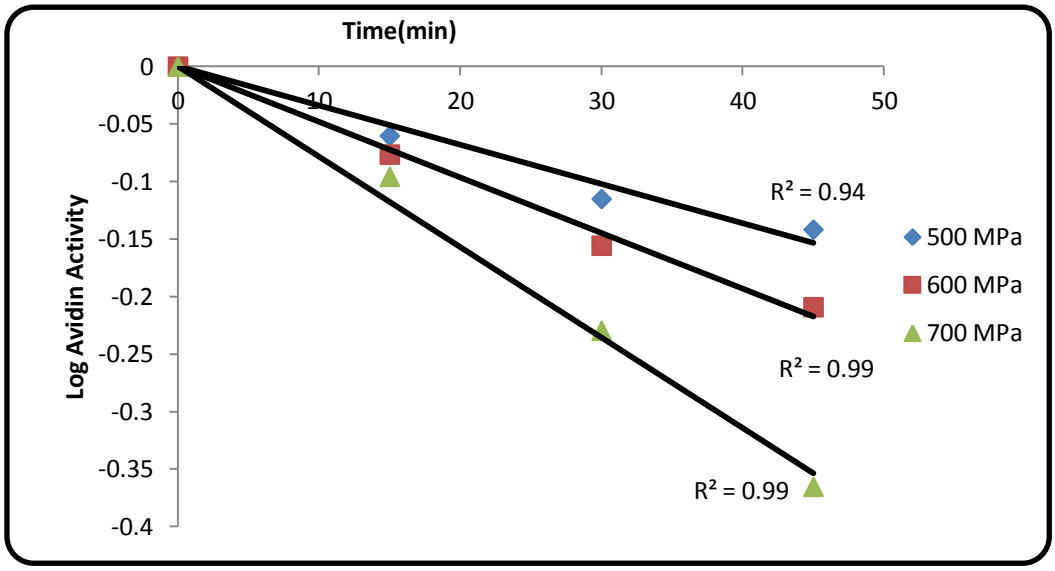


Figure 3.4: Effect of HP-HT on inactivation of avidin at 80°C

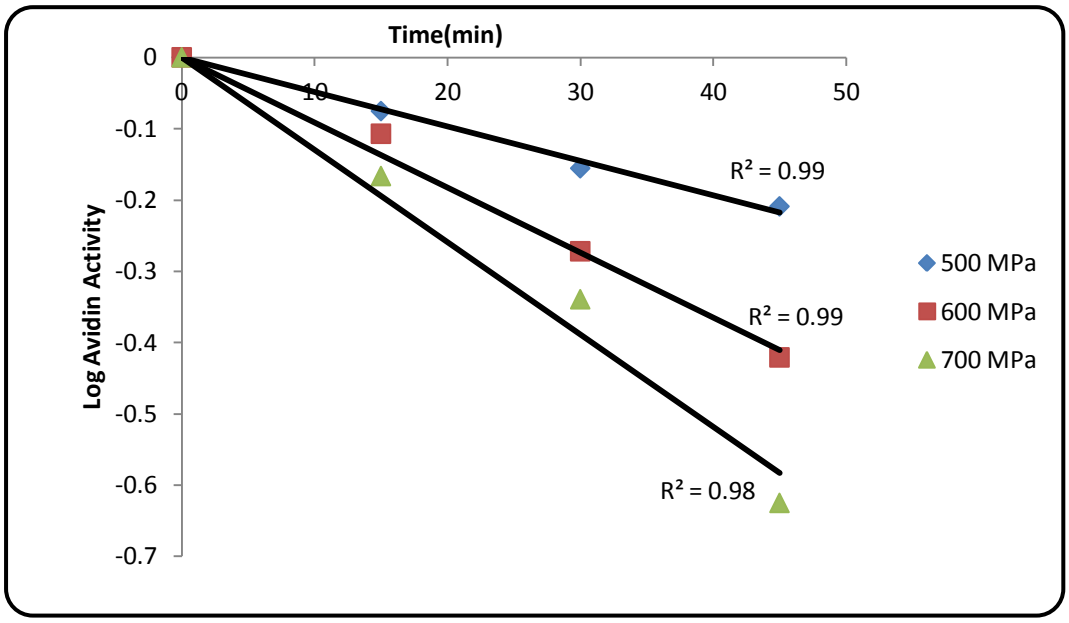


Figure 3.5: Effect of HP-HT on inactivation of avidin at 90°C

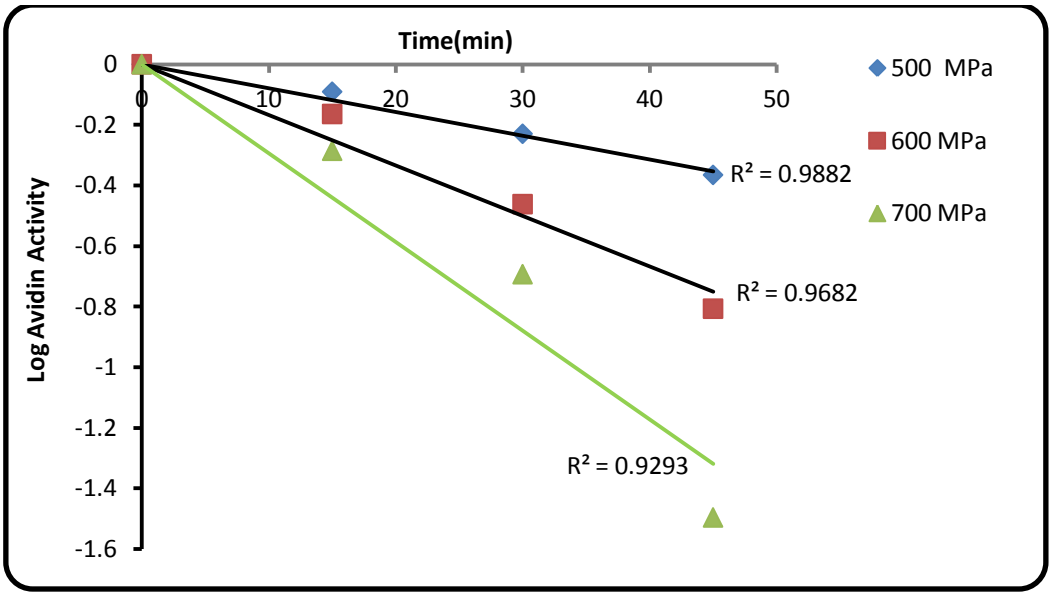


Figure 3.6: Effect of HP- HT on inactivation of avidin at 100°C

Based on TDT and PDT model, z_T and z_P of avidin were analyzed. Thermal resistance at constant pressure was well described by z_T value concept. Thermal resistance (Z_T) was determined at each pressure with help of regression analysis and shows decrease in z value with increasing treatment intensity (Figure 3.7). The corresponding Z_T values were 47.4°C, 42.4°C, 33.9°C and 54.3°C at 500, 600, 700 and 0.1MPa, respectively (Table 3.6). z value with thermal treatment was found to be 49.6°C (Table 3.3) which is higher than obtained from HPHT treatments. This inferred that avidin activity is more sensitive to temperature changes under pressure than thermal treatments alone.

Table 3.6: High pressure and temperature combinations destruction of avidin in 0.02 M sodium phosphate buffer (pH 7.3)

Pressure (MPa)	Temperature (°C)	D value	R^2	Z value (°C)	R^2
500	80	322	0.96		
	90	212	0.99	47.4	0.99
	100	122	0.99		
600	80	178	0.99		
	90	109	0.99	42.4	0.99
	100	60.2	0.95		
700	80	132	0.98		
	90	76.9	0.98	33.9	0.99
	100	33.9	0.93		
0.1	80	333	0.99		
	90	238	0.99	54.3	0.99
	100	143	0.98		

Table 3.7: High pressure Z_P value of avidin inactivation in sodium phosphate buffer

Pressure (MPa)	Z_P value (MPa)	R^2
500	526	0.97
600	455	0.98
700	345	0.99

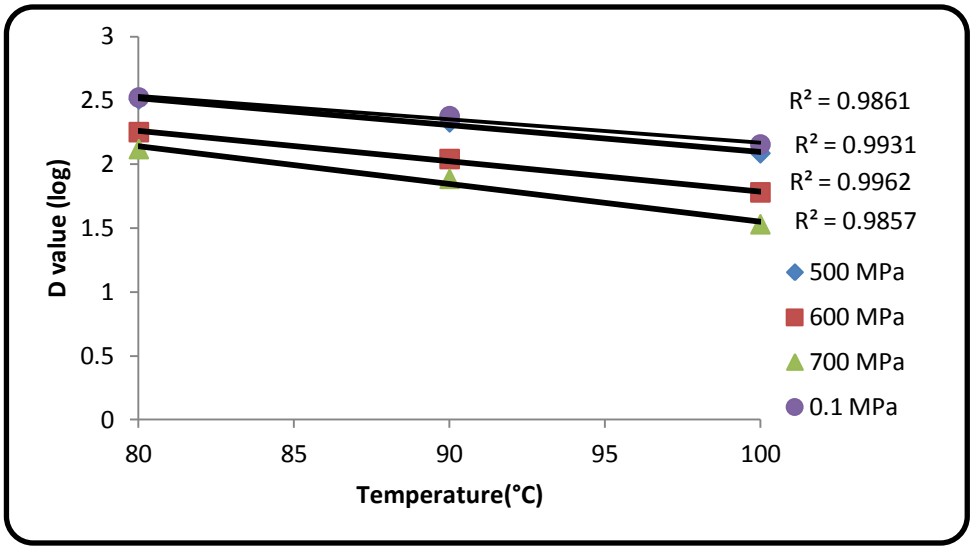


Figure 3.7: Relationship between temperature and logarithmic D value of avidin (high pressure high temperature treatment combination and thermal treatment)

In this study, the high pressure high temperature combination was able to cause 99% destruction of avidin activity. In similar work, it was reported that inactivation of PME (pectin methyl esterase) in orange juice was more than 90%, and the extent of inactivation was dependent on pressure level, pressure holding time, pH level and soluble solids (Basak and Ramaswamy, 1996). HPP was found to cause reduction in activity of enzymes in food with increase in strength of high pressure treatment (Hendrickx et al., 1998; Riahi and Ramaswamy, 2003; Juan et al., 2007).

3.4.2.2 HPHT inactivation kinetics of raw egg samples

Inactivation kinetics of avidin activity in raw egg at different pressures (500, 600 and 700 MPa) in combination with temperature (30, 40 and 50°C) as function of pressure holding time for EW and WLE has been detailed in Table 3.8. During pressure holding time, data was well fitted to first order model. High pressure and temperature combination caused further rapid inactivation of avidin activity as shown from resulting D and z values which were lower at low pressure level and low temperature combination treatments (Table 3.8 and 3.9). Similar conclusions were drawn by Balny and Masson

(1993) in their study stating that high pressure in combination with moderate temperature causes denaturation of proteins. While comparing HPHT (High Pressure-High temperature) to thermal treatment, it was recognized that HPHT was more efficient for avidin inactivation in case of both EW and WLE. In thermal treatments, corrected D value of 9.38 and 8.73 min were achieved for EW and WLE respectively at 80°C (Table 3.4). On other hand, when high pressures of 700 MPa at much lower temperature of 50°C was used, it resulted in D value of 2.18 and 2.22 min which is almost 5 times lesser than that of thermal treatment (Table 3.8).

Table 3.8: Decimal reduction time (D values) of avidin activity with HP treatment for a) Egg white b) Whole liquid egg

(a)

Pressure (MPa)	D value (30°C)	R^2	D value (40°C)	R^2	D value (50°C)	R^2
500	19.5	0.974	10.4	0.944	7.46	0.986
600	8.70	0.984	5.09	0.945	3.21	0.965
700	6.65	0.979	3.26	0.953	2.18	0.912

(b)

Pressure (MPa)	D value (30°C)	R^2	D value (40°C)	R^2	D value (50°C)	R^2
500	23.6	0.995	11.0	0.911	7.88	0.984
600	10.3	0.971	5.28	0.932	3.31	0.959
700	7.48	0.986	3.36	0.944	2.22	0.909

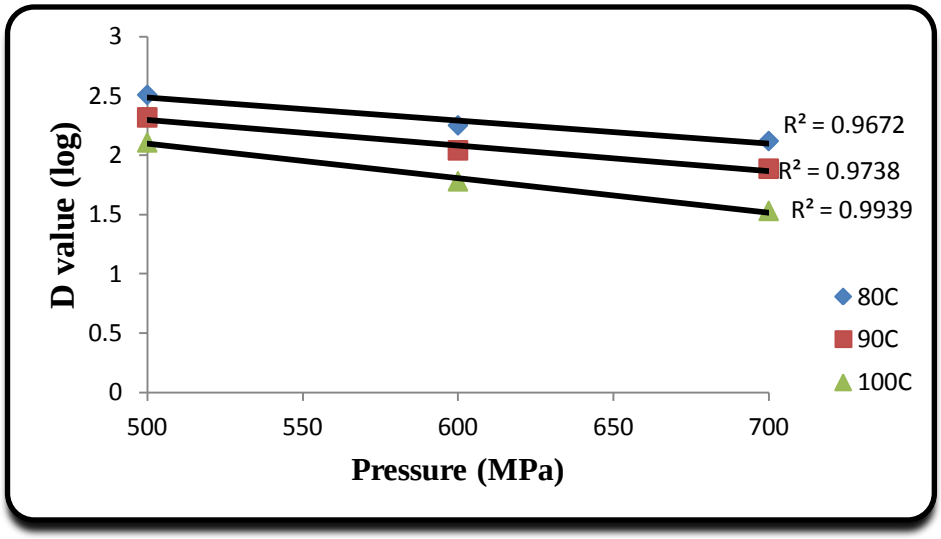


Figure 3.8: Relationship between pressure and logarithmic D value of avidin

D values as function of pressure or temperature were analyzed to obtain Z_T and Zp of avidin in egg white and whole liquid egg. For egg white, Z_T values were 47.4, 46.1 and 41.2°C and for whole liquid egg, it was 44.2, 42.2 and 39.8°C at 500, 600 and 700 MPa (Table 3.9) which is lower than 43.3°C (Egg white) and 48.1°C (Whole liquid egg) at atmospheric pressure (Table 3.6).

Table 3.9: a) Z_P and b) Z_T value of egg white and whole liquid egg from different pressure treatments at different temperature.

(a)	Egg White		Whole liquid egg	
Temperature	**Z_P value**	**R^2**	**Zp value**	**R^2**
30 °C	417 MPa	0.923	400 MPa	0.937
40 °C	400 MPa	0.982	385 MPa	0.981
50 °C	370 MPa	0.956	370 MPa	0.955

(b)	Egg White		Whole liquid egg	
Pressure	**Z_T value**	**R^2**	**Z_T value**	**R^2**
500	44.4 °C	0.974	47.4°C	0.958
600	42.2 °C	0.998	46.1°C	0.993
700	39.8 °C	0.975	41.2°C	0.972

Pressure resistance (Z_P) was obtained from correlation between logarithmic D values and pressure for each temperature. Z_P (pressure resistance) value of 417, 400 and 370 were obtained for egg white and 400, 385 and 370 at 30, 40 and 50°C for whole liquid egg which inferred that avidin activity diminished with increase in pressure level. It appears that avidin is more sensitive to small temperature changes under high pressure.

These results depicts that high pressure in combination with temperature (30-50°C) can inactivate avidin activity and effectively shorten the processing time. Thus, HPP can be used to minimize quality losses and process cost for egg and egg products as compared to thermal processing. Nienaber and Shellhammer (2001) studied effect of high pressure (400-600 MPa) and temperature (25-50°C) for various holding times which resulted in PME inactivation. Pandey and Ramaswamy (2004) found that increasing HP treatment resulted in rapid inactivation of glutamyl transferase, following a first order kinetic model. Activity was found to range from 45% at 300 MPa and 19% at 350 MPa to just 3% at 400 MPa. They found that with pressure in excess of 500 MPa, faster inactivation rates were obtained hence, resulting in economical viability of process. Shook et al., 2001 revealed that HPP (400-800 MPa) in combination with temperature (25 and 50°C) for 1, 3 and 5 min causes significant effect on inactivation of lipoxygenase and polygalacturonase (PG) in tomato, with complete loss of PG activity at 800 MPa.

Overall, it was found that pure avidin showed an order of magnitude higher resistance to its inactivation by both thermal and high pressure; however high pressure caused a 4 times faster inactivation of pure avidin than thermal treatments. On other hand, avidin present in the egg showed very little stability to thermal and pressure treatments. Thermal treatment of 100°C for 10 min was able to cause inactivation of avidin present in the egg. Even in this case, pressure treatment caused faster inactivation of avidin present in the egg. Pressure treatment of 700 MPa/50°C/ 10 min caused inactivation of avidin present in the egg. This way pressure treatment has advantage over thermal treatment that it can cause inactivation of avidin in egg at lower temperature thus preserving quality of egg proteins from heat damage. The main reason for improved

inactivation by high pressure is that it causes compression of proteins in reversible or irreversible manner and thus affecting their tertiary and quaternary structure. Protein unfolding at low temperature could be possible due to compressibility of molecule which is evident from changing spatial positions of secondary structure domains and pressure induced exposure of hydrophobic groups located in interior of protein (Knorr et al., 2006).

3.5 Conclusions

In this study, the effect of HP and temperature combinations and thermal treatment was evaluated for inactivation of avidin activity. The effect of temperature on inactivation of avidin is important for high pressure processing applications in food industry. Pure avidin showed higher resistance to inactivation by thermal treatments. High pressure when used along with high temperature treatments (700 MPa/100°C) gave D value of 33.9 min which was less than that of highest thermal treatment used (100°C) i.e. $D_{100^{\circ}C}$ of 143 min in pure avidin. Similarly for avidin activity inactivation in raw EW and WLE, D values of 2.18 and 2.22 min were found which were way less than $D_{100^{\circ}C}$ =10.8 and 10 min respectively. This data shows avidin naturally present in egg is sensitive to inactivation by thermal temperature, but in grouping with high pressure, faster inactivation rate is obtained. This study demonstrates advantageous effect of HP treatment on inactivation of biotin binding activity of avidin and implies that pressure and temperature combination inactivation data for avidin would be useful tool for establishing HP treatment process.

PREFACE TO CHAPTER 4

In Chapter 3, it was shown that high pressure processing can be used to inactivate anti-nutritive enzymes in food. At this pressure level (up to 600 MPa), there are significant changes in structural and functional properties of egg components. From process establishment point of view, it is imperative to evaluate quality and the extent of rheological changes caused by HPP. Rheological properties are studied worldwide to know about consistency, degree of fluidity, and other mechanical properties that allow us to understand food shelf stability and determine food texture.

In this part, rheological characteristics of various egg components were studied at various pressure levels. Optimization was performed to find pressure and time-temperature combinations in which egg components can be kept in liquid flowing stage. Composite central design was used to cover widest possible range of process variable and their effect on rheological characteristics.

Part of this research was presented in annual meeting of institute of food technologists, New Orleans, USA in 2011.

Singh A and Ramaswamy HS (2012) Evaluation of high pressure processing effects on the rheological properties of egg components using response surface methodology (Prepared for Submission).

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy.

CHAPTER 4

EFFECT OF HIGH PRESSURE PROCESSING ON THE RHEOLOGICAL PROPERTIES OF EGG COMPONENTS USING RESPONSE SURFACE METHODOLOGY

4.1 Abstract

The focus of the study was to see the effect of high pressure processing on rheological characterization of different egg components i.e. egg white, egg yolk and whole liquid egg. A central composite design with three independent variables namely pressure (281.8, 350, 450, 550 and 618.2 MPa); temperature (8.2, 15, 25, 35 and 41.8°C); holding time (1.6, 5, 10, 15 and 18.4 min) was used for the current study. Samples were subjected to preset shear rate increasing linearly from 0-100 s^{-1} in 5 min (upward curve), followed by steady shear at 100 s^{-1} for 5 min (Hold curve), and finally decreasing shear rate from 100 - 0s^{-1} for 5 min (downward curve) using parallel plate advanced rheometer. Power law was fitted to upward and downward curve and Weltman model fitted well for the hold curve (R^2 >0.90). All egg components showed thixotropic behavior with time but use of high pressure processing reduced time dependency. Pressure was the most significant factor followed by time and temperature for all egg components. Egg components showed transition from liquid (218-350MPa)-semi viscous (350-500Mpa) - highly viscous (500-618MPa) by an increase in pressure treatment. Flow behavior index and consistency coefficient varied significantly for all egg components.

4.2 Introduction

An increase in demand of high quality products with minimal processing, without the use of additives and preservatives has offered a fertile ground for the non-thermal techniques of food processing to establish its own niche in the global markets. High pressure processing (HPP) is one such emerging non-thermal alternative to conventional food processing techniques for producing high quality foods. It works on the principle of

application of high pressures to attain shelf stable products and to modify the functional properties of food systems by mildly altering the protein structures, flavor and nutritional qualities. The isostatic rule which governs HPP states that pressure is instantaneously and uniformly transmitted throughout a sample. Therefore, in contrast to conventional thermal processing, the time necessary for HPP is independent of the sample size (Rastogi et al., 2007). HPP has shown to inactivate spoilage causing micro-organisms and enzymes (Alderton et al., 1976; Oxen and Knorr., 1993; Arroyo et al., 1997; Mussa et al., 1999; Denys et al., 2000) resulting in a product of desired quality. Its application can contribute towards improvement of the physico chemical properties like color, gel characteristics and water holding capacity of the food systems (Johnston et al., 1993). HPP has shown to cause a 7-$\log_{10}$ reduction of *S. enterica* serovar *enteritidis* in liquid whole eggs (Ponce et al., 1998). Denaturation of various protein systems, changes in functionality (Lopez-fandino et al., 1996), structural modifications (Van Camp et al., 1995) are some of objectives which can be achieved by using this technique.

The egg has high protein content, which undergoes denaturation or structural changes such as coagulation on the application of heat or pressure in case of HPP. Any changes in the structure of protein can affect the rheological behavior of the food systems such as gel and emulsions to a great extent hence it would be exciting to explore the flow properties of HP treated egg (Hayakawa et al., 1996).

Rheological characteristics act as a support index to establish the optimized values for transportation of liquid or semi-solid foods throughout the processing line (Ahmed et al., 2003). The consistency, degree of fluidity, and other mechanical properties are important in understanding how long food can be stored, how stable it will remain, and in determining food texture.

Being a complex system both structurally and compositionally, foods cover a broad range of different rheological behaviors such as pseudo plastic; shear thickening, thixotropic, and viscoelastic (Rao, 1986). Food is pasteurized mainly using thermal processing techniques which may alter its coagulation; foaming and emulsifying properties. HPP has several advantages over thermal processing like better functional properties, no post processing contamination(in container treatment) and better

preservation of nutritional quality as it does not require high temperature (Ahmed et al., 2003). Use of HPP for denaturation of protein has always been an exciting concept as it can cause modification of functional properties by stimulating protein conformational changes. It can modify the functional properties of food components without affecting other characteristics, thus satisfying increased demand of minimally processed high quality foodstuff. HPP can highly affect protein concentration of egg and the impact of HP on aggregation and network formation can also be modulated by pH (Aguilar et al., 2007). Hence, the analytical study of impact of HPP on rheology has become an attractive and essential area of research for scientists. The present investigation was undertaken to evaluate the changes in rheological characteristics of various egg components with the application of HPP to evaluate phase changes from liquid to semi solid and partially formed gel in various egg components.

4.3 Materials and methods

4.3.1 Sample preparation

Raw eggs were obtained from a local grocery store in Sainte-Anne-de- Bellevue, QC (Canada) not older than 10 days after being laid down. Only large A size eggs were taken for all experiments. They were sorted to exclude any cracked or inferior quality eggs. The eggs were mixed well to obtain a sample of whole liquid egg. For individual use of albumen and egg yolk, eggs were carefully broken from the top and egg white was allowed to pour slowly leaving the yolk behind. Separated egg yolk and egg white were individually packed in flexible polyethylene pouches for the processing treatments. Sample preparation was immediately followed by pressure treatment.

4.3.2 High hydrostatic pressure treatment

HP treatments were carried out in an isostatic press (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France) with a cylindrical pressure chamber of capacity 5L. A high temperature circulating water bath (VWR Model 1197) was connected to HP chamber in order to control the temperature during the processing. The pressurization medium used was pure water. The flexible pouches containing whole liquid egg, egg yolk

and egg white (WLE, EY and EW) were submerged in water at a desired temperature for the pressurizing treatment. The pressure build up or releasing time was not considered during the treatment time. Pressurization and depressurization rate of 4.4 MPa/s and 26 MPa/s respectively, were used during high pressure treatments. The pressure treated pouches containing the sample were immediately transferred to refrigerator (4°C) followed by rheological analysis. Duplicate measurements were carried out for each run for all the samples.

4.3.3 Post treatment evaluation

A controlled stress rheometer (AR 2000, TA Instruments, New Castle, DE, USA) with attached computer software (Rheology Advantage Data Analysis Program, TA version 2.3 s) was used for all the rheological measurements for WLE, EW and EY. Parallel plate geometry (60mm, 1mm gap) was used to measure the flow behavior of egg samples with the instrument programmed at 20°C. Zero gap and instrument rotational mapping was efficiently performed before executing the experiments so as to get reliable results. 2 mL sample of each WLE, EY or EW was transferred to the flat plate of the rheometer. Every test comprised of three cycle shear phases from an upward curve at 0 - 100s^{-1} to a holding period at 100 s^{-1} for 5 min followed by a downward curve from 100 - 0 s^{-1} in 5 min.

4.3.4 Experimental design

The design was used to estimate the effect of independent variables such as pressure (X_1); treatment time (X_2) and temperature (X_3) on the dependent variables such as flow behavior index (n) and consistency coefficient (m) for upward and downward flow curve, and constant A and B for hold curve of the various egg components (EW, EY, WLE). Twenty combinations of independent variables were selected by experimental design for three parameters as shown in Table 4.1. Quadratic polynomial regression model was used for correlating these values to their coded variables (x_i, i = 1, 2, and 3).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (4.1)$$

where b_0 (constant term), b_1, b_2 and b_3 (linear effects), b_{11}, b_{22} and b_{33} (quadratic effects), and b_{12}, b_{13} and b_{23} (interaction effects) represents the coefficients of the polynomial model.

Table 4.1: Minimum and maximum levels of process variables (pressure, temperature and holding time)

CCRD levels	Pressure (X_1) (MPa)	Temperature (X_2) (°C)	Holding time (X_3) Min
-1.68	281.8	8.2	1.6
-1	350	15	5
0	450	25	10
1	550	35	15
1.68	618.2	41.8	18.4

The number of experimental points in the CCRD was sufficient to test statistical validity of the fitted model and lack-of-fit of the model (Arteaga et al., 1994). The central point in CCRD was replicated several times to estimate the error due to experimental or random variability. The process was optimized for two independent variables at a time using RSM.

4.3.5 Modeling

Logarithmic plots of shear stress versus shear rate data of treated egg samples used to calculate n and m values (Taherian et al., 2007). Data was tested for various rheological models but power law model fitted adequately for upward and downward curves (based on the determination coefficient ($R^2 > 0.90$) among the different models at all test conditions).

Power law is presented as:

$$n = m\gamma^{n-1} \tag{4.2}$$

"m" is the consistency coefficient ($Pa.s^n$) which describes the overall viscosity range of

the flow curve. The flow behavior index is denoted as "n" and it is a dimensionless quantity.

4.3.6 Time dependency behavior

In order to investigate thixotropy in high pressure processed egg products, steady shear rate of 100 s^{-1} for 5 min (hold curve) was applied. Stress decay behavior for all conditions was well explained by modified Weltman model (1943) as expounded by Basak and Ramaswamy (1994). The constant A and B were obtained from the regression of (σ) versus (log t).

Weltman equation is given as following:

$$\sigma = A - B\left[\left(\log\frac{t}{t_m}\right)\right] \tag{4.3}$$

where $t > t_m$, σ = shear stress, t = time (sec), t_m = time at maximum observed shear stress, A = intercept when log $(t/t_m) = 0$ or $t = t_m$ (pa), B= slope (time coefficient of thixotropic breakdown) (pa)

4.3.7 Statistical analysis

A central composite design for variables was selected and the co-ordinate was given by factorial design. Regression coefficients and ANOVA Table was computed using Design Expert Software. Surface graphs were plotted for the predicted values obtained from the models against two different process variables. The models were analyzed for coefficient of determination and standard error.

4.4. Results and discussions

A central composite rotatable design (CCRD) was used for designing experiments as shown in Table 4.1 and rheological characteristics were measured and second order polynomial model was suggested for response variables by CCRD model.

Power law (Eq 4.2) was used to calculate the consistency index (m) and flow behavior index (n). These two parameters, m and n were used in depth to build understanding of the role of protein structure on rheology of egg components. Weltman

model was used for the time dependent viscosity changes at a steady shear during the hold time. The range of consistency index and flow behavior index of upward and downward flow curves, as well as the Weltman parameters, of various egg components are shown in Table 4.2. ANOVA analysis was used to fit "n" and "m" values to second order polynomial equations. Sum of squares of sequential model was evaluated for model fitting. The scope was to test pressure in combination with time and temperature so as to study changes in egg components over broader range (complete liquid to partial gel state). The suitability of the fitted functions was evaluated by the coefficient of determination (R^2).

Table 4.2 Range of n and m values of upward, downward flow and A and B constant of hold curves (egg components)

Source	n value (-)	m value (Pa s^n)	Constant A	Constant B
EW up curve	0.19-0.52	0.41-4.4		
EW hold curve			1.01-1.18	5- 8.1
EW down curve	0.18 - 0.79	0.023-14.6		
EY up curve	0.097 -0.11	31.2 -122.9		
EY hold curve			24.5 -441	279 -721
EY down curve	0.129 - 0.68	11.1 - 33.5		
WLE up curve	0.15- 0.36	1.2- 41.2		
WLE hold curve			3.7 -15.2	9.2 -70.2
WLE down curve	0.14-0.83	0.05 -7.93		

4.4.1 Experimental data handling

For evaluation of statistical significance of terms, regression coefficients and ANOVA analysis were used. For each response variable, regression coefficients of model and the coefficients of determination (R^2) were calculated. Lack of fit was assessed and found to be insignificant, thus indicating model used was accurate. Lack of fit was used to evaluate integrity of regression model whether model is correct or not (Montgomery, 2001).

4.4.1.1 Effect of process variables on flow characteristics of egg white

From the statistical analysis of variance (ANOVA), it was found that all independent variables - pressure, temperature and holding time, had a major influence on all rheological parameters of egg white (Table 4.3 and 4.4).

Table 4.3: Flow behavior index (n) and consistency coefficient (m) for upward and downward curve; constant A and B for hold curve of egg white

No.	Pressure	Time	Temp.	Up curve		Hold Curve		Down Curve	
	MPa	min	°C	n	m	A value	B value	n	m
1	350(-1)	15 (1)	35 (1)	0.316	1.857	1.01	5.00	0.591	0.550
2	450(0)	10 (0)	25 (0)	0.52	1.03	0.52	7.23	0.545	2.382
3	450(0)	10 (0)	25 (0)	0.519	1.004	1.01	8.63	0.545	2.382
4	350(-1)	5(-1)	15 (-1)	0.216	2.358	1.25	6.49	0.795	0.226
5	550(1)	5(-1)	35 (1)	0.523	1.165	0.72	9.29	0.386	8.820
6	550(1)	15 (1)	15 (-1)	0.359	3.831	5.14	12.34	0.401	7.437
7	350(-1)	5 (-1)	35 (1)	0.32	1.862	2.06	2.35	0.657	0.269
8	450(0)	10 (0)	25 (0)	0.52	1	0.20	7.74	0.551	2.320
9	550(1)	15 (1)	35 (1)	0.212	4.322	0.91	18.19	0.184	14.60
10	550(1)	5 (-1)	15 (-1)	0.524	0.863	0.87	7.50	0.574	1.663
11	350(-1)	15 (1)	15 (-1)	0.332	1.469	0.35	3.87	0.799	0.235
12	450(0)	10 (0)	25 (0)	0.52	1.003	0.07	8.37	0.556	2.336
13	450(0)	10 (0)	8.2 (-1.68)	0.481	0.414	0.77	4.72	0.642	0.023
14	450(0)	10 (0)	25 (0)	0.52	1.005	1.03	8.17	0.482	2.353
15	450(0)	18.4(1.68)	25 (0)	0.19	2.997	1.23	9.74	0.386	5.190
16	618.2(1.68)	10 (0)	25(0)	0.31	4.425	6.13	19.22	0.199	13.29
17	450 (0)	10 (0)	41.8(1.68)	0.519	0.862	0.43	4.74	0.333	6.229
18	281.8(-1.68)	10(0)	25 (0)	0.219	4.03	4.31	8.17	0.749	0.096
19	450(0)	1.59(-1.68)	25 (0)	0.336	0.745	0.77	4.14	0.574	0.312
20	450(0)	10 (0)	25 (0)	0.477	1.157	1.18	8.11	0.480	2.420

Table 4.4: ANOVA analysis and regression coefficient of second order polynomial model for response variables (upward, hold and downward curve) for egg white

Upward		Flow behavior Index			Consistency Coefficient		
	Source	Sum of Squares	DF	Prob> F	Sum of	DF	Prob > F
	Block	0.0031	2		0.139	2	
	Model	0.2990	9	< 0.0001	32.53	9	< 0.0001
Linear	P	0.0252	1	0.0002	0.797	1	0.0004
	T	0.0271	1	0.0001	5.956	1	< 0.0001
	T	0.0000	1	0.9492	0.152	1	0.0342
Quadratic	P^2	0.1007	1	< 0.0001	17.618	1	< 0.0001
	t^2	0.1021	1	< 0.0001	1.073	1	0.0001
	T^2	0.0000	1	0.9626	0.383	1	0.0037
Interaction	P*t	0.0432	1	< 0.0001	6.159	1	< 0.0001
	P*T	0.0070	1	0.0081	0.102	1	0.0704
	t*T	0.0088	1	0.0044	0.144	1	0.038
	Residual	0.0046	8		0.187	8	
	Lack of Fit	0.0037	5	0.2497	0.175	5	0.0524
	R-Squared	0.9850			0.994		
Hold Curve		**Constant A**			**Constant B**		
	Block	5.35	2		0.5	2	
	Model	47.35	9	< 0.0001	347.1	9	< 0.0001
Linear	P	2.67	1	0.0005	170.1	1	< 0.0001
	T	0.78	1	0.0158	39.4	1	< 0.0001
	T	0.88	1	0.012	1.6	1	0.0735
Quadratic	P^2	29.08	1	< 0.0001	53.1	1	< 0.0001
	t^2	0.07	1	0.3823	3.2	1	0.0197
	T^2	0.64	1	0.0246	22.5	1	< 0.0001
Interaction	P*t	5.13	1	< 0.0001	23.5	1	< 0.0001
	P*T	4.28	1	< 0.0001	14.2	1	0.0003
	t*T	2.23	1	0.0009	10.9	1	0.0006
	Residual	0.67	8		3.0	8	
	Lack of Fit	0.53	5	0.2689	1.8	5	0.5593
	R-Squared	0.99			0.99		
Down Curve		**Flow Behavior Index**			**Consistency Coefficient**		
	Block	0.022	2		0.1016	2	
	Model	0.535	9	< 0.0001	358	9	< 0.0001
Linear	P	0.362	1	< 0.0001	209	1	< 0.0001
	t	0.041	1	< 0.0001	29	1	< 0.0001
	T	0.118	1	< 0.0001	46	1	< 0.0001
Quadratic	P^2	9.09E-05	1	0.0039	33	1	< 0.0001
	t^2	5.55E-06	1	0.3518	0.205	1	< 0.0001
	T^2	6.91E-05	1	0.0082	0.914	1	< 0.0001
Interaction	P*t	0.0122	1	< 0.0001	16	1	< 0.0001
	P*T	0.0004	1	< 0.0001	24	1	< 0.0001
	t*T	0.0012	1	< 0.0001	0.0097	1	0.0159
	Residual	4.54E-05	8		0.0083	8	
	Lack of Fit	3.10E-05	5	0.445	0.0060	5	0.3874
	R-Squared	**1**			**1**		

*P=pressure, t= time, T=temperature.

Flow behavior index showed increment with increase in pressure and time. Lack of fit was found to be insignificant. Except temperature for the hold curve, the process variables significantly influenced the rheological parameters and their interactions (p<0.05) (Table 4.4).

Pressure treatments caused significant changes in the flow behavior index. In the up curve, there was variability in n and m values. The possible reason for variability in n & m value was due to reason that egg white samples subjected to up curve shear were virgin and showed lot of unevenness in their values. The time effect favored a slight increase in flow behavior index at the lower pressure levels while it tended to do the reverse at higher pressures (increasing the pseudo-plasticity of the samples). Pressure level was more pronounced for when the treatment times were short (5 min) as compared to longer (15 min) treatment times.

The consistence coefficient (m value) was influenced in a more systematic way by the process variables. The temperature influence was mild, while treatment time resulted in a steady increase in the consistency coefficient of the egg white. Pressure-temperture curve showed a slight concave dip at mid level of pressure (Figure 4.1).

High pressure is known to change stability of protein complex which cause denaturation and in turn unfolding of proteins. Pressure induced denaturation is a complex process which is entirely dependent on protein type, pH, ionic strength and composition of food product (Hayashi et al., 1989). High pressure affects protein conformation and leads to denaturation or gelation depending on the protein system and treatment conditions (Messens et al., 1997).

In the hold curve**,** thixotropic behaviour was examined using the modified Weltman logarithmic model and a good fit was obtained. In hold curve, constants A and B were calculated using Weltman equation. These constants explain about effect of constant shearing on treated samples.

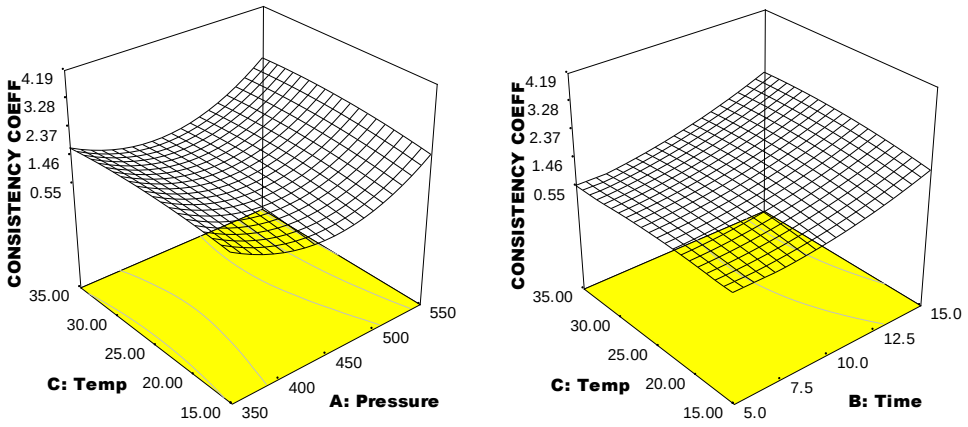


Figure 4.1: Three dimensional (3D) response surface plots presenting effect of HPP, time and temp. on the consistency coefficient (m value) of upward curve for EW

Weltman constant A has close correlation with yield stress as it is a measure of initial resistance to shear rate at time equal to 1s. It was significantly affected by linear effect of pressure (p<0.05), quadratic effect of pressure (p<0.05), interaction of pressure*time (p<0.05) and pressure*temperature (p<0.05) (Table 4.4). Constant A was most affected by pressure followed by temperature and time. Pressure caused the linear increase in value of constant A, whereas with increase in temperature and time, value of constant A remained constant throughout (Figure 4.2).

Weltman constant B is a measure of the rate of structure breakdown referred to as time coefficient of stress decay. Higher structure loss is indicated by higher value of constant B. Constant B was significantly (p<0.05) influenced by linear, quadratic and interaction effects of pressure, time and temperature except linear effect of temperature (p<0.05) Increase in pressure and time caused linear increase in the constant B value followed by sharp increase, but the B value showed an initial increase followed by constant values (Figure 4.3).

It was found that high pressure can cause changes in functional properties like solubility and structural characteristics of ovalbumin which is a major constituent of egg white. Ovalbumin showed high sensitivity to high pressure which can cause increase in viscosity due to unfolding of proteins but they retain foaming and other functional properties (Lametti et al., 1998).

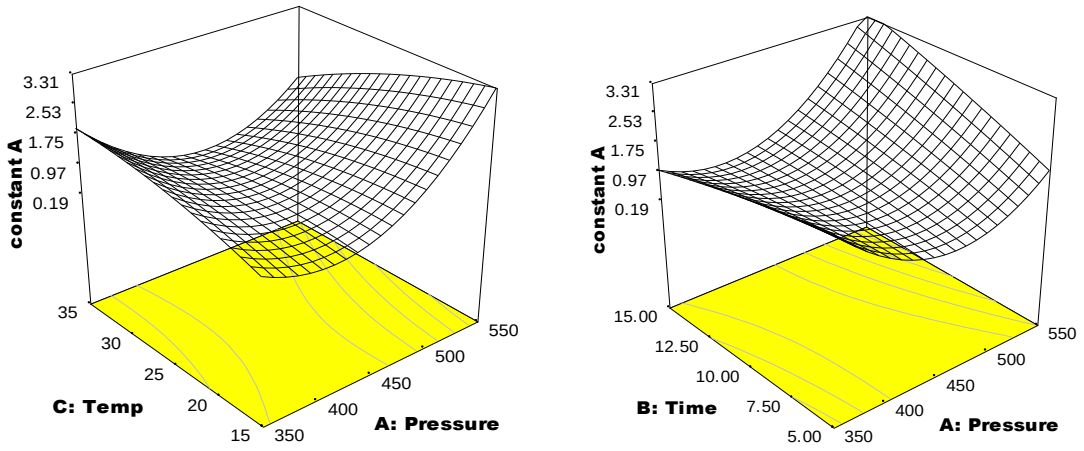


Figure 4.2: Three dimensional (3D) response surface plots presenting effect of high pressure processing, time and temperature on the Constant A of hold curve for egg white

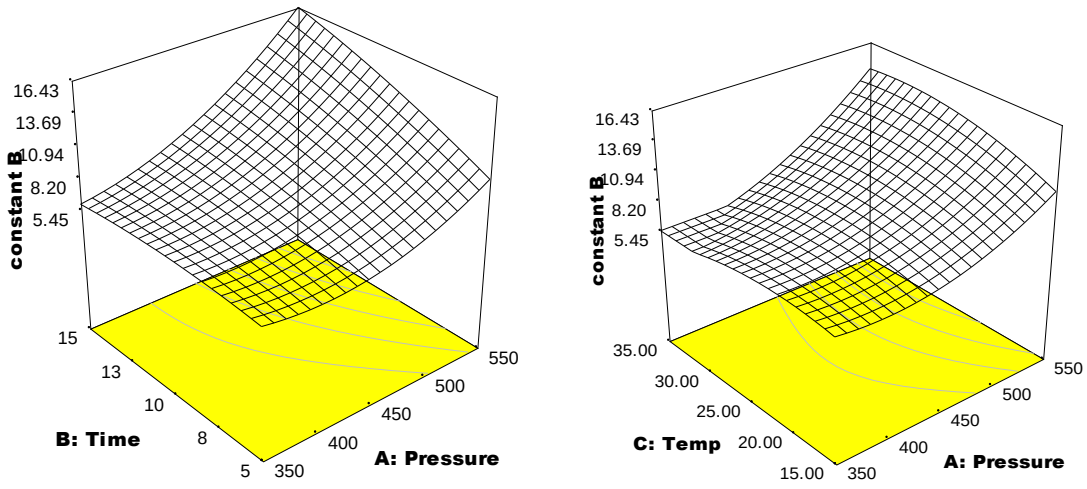


Figure 4.3: Three dimensional (3D) response surface plots presenting effect of high pressure processing, time and temperature on the Constant B of hold curve for egg white.

In downward curve, shear stress of 100 - 0 s^{-1} was applied over time period of 5 min. While the up-curve rheology simulates the behavior of virgin samples (not subjected to any shear), the down curve rheological parameters are for stirred sytems and are more useful in flowing liquids. Similar to that of upward curve, n value was less than 1 showing pseudo-plastic behavior. The n value remained constant with increase in treatment time, whereas increase in pressure and temperature caused decrease in the n value (Figure 4.4). For m value, model (p<0.05), linear effect of P, t and T (p<0.05) quadratic effect of P, t and T (p<0.05) and interaction effect of P*t and P*T were highly significant (p<0.05). Pressure caused increase in m value with increase in pressure, whereas with increasing holding time and temperature, m value remained constant throughout (Figure 4.5). The m value was more significantly influenced by pressure in comparison to time and temperature for downward curve.

From rheology and visual appearance, the egg white was found to give liquid appearance up to pressure-temperature treatment (450MPa/25°C/1.6min) resulting in flow behavior index of 0.336. After this treatment, egg white had higher viscosity not allowing it to flow freely. From pressure-temperature treatment of (450MPa/25°C/10min) onwards having n value of 0.519, the viscosity of egg white increased resulting in semi viscous egg gels. Hayashi et al. (1989) evaluated effect of high pressure on albumen and egg yolk and found that high pressure treatment (400 MPa/25°C/30 min) caused less gelation than cooking in water bath for 2-10 min.

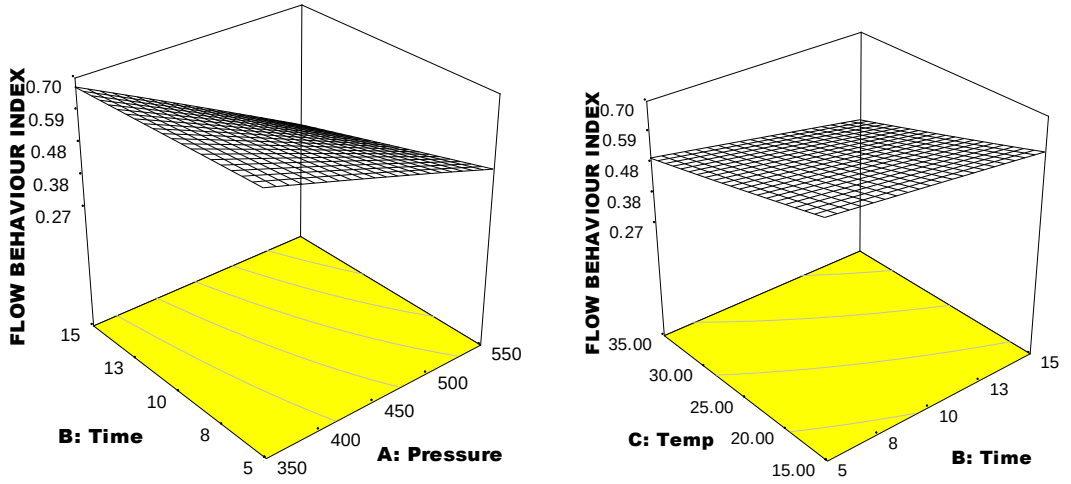


Figure 4.4: Three dimensional (3D) response surface plots presenting effect of high pressure processing, time and temperature on the flow behavior index (n value) of downward curve for egg white

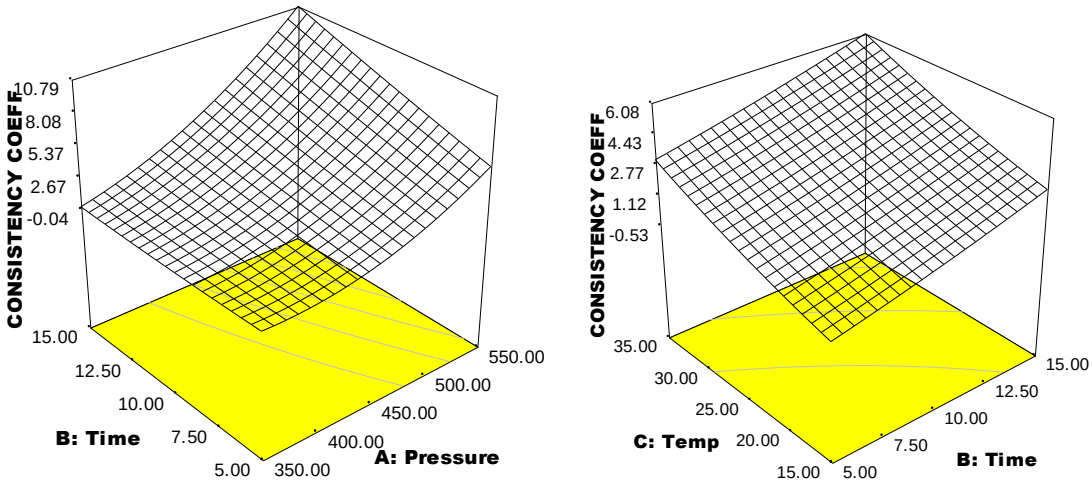


Figure 4.5: Three dimensional (3D) response surface plots presenting effect of high pressure processing, time and temperature on the consistency coefficient (m value) of downward curve for egg white

4.4.1.2 Effect of process variables on flow characteristics of egg yolk

It was found that all process variables (pressure, temperature and holding time) had significant effect on the n and m value of egg yolk (Table 4.5). The model was found to be significant for upward, downward and hold curves of egg yolk.

Up curve: Shear stress of 0-100 s^{-1} was applied over a time period of 5 min for n value. The values of flow behavior index (n) were less than 1, indicating the shear thinning nature of the egg yolk at different processing conditions. In similar study, it was found that the egg yolk shows slightly pseudoplastic behavior at different total solids content (Scalzo et al., 1970). Analysis indicated that pressure (p<0.05) had most significant effect on n value followed by time ((p<0.05) and temperature (p<0.05) (Table 4.6). The lack of fit was not significant, implicating accuracy of model for predicting the response (Myers and Montgomery, 2002). Model fitting and ANOVA were validated by analyzing residuals, including the examination of diagnostic plots and calculation of case statistics. The response surface was generated by keeping one variable at its zero level (center point) and varying the others in their experimental range.

On the other hand, the value of consistency coefficient (m value) increases for upward curve, pressure and time causing significant changes in consistency coefficient. Linear, quadratic and their interaction effects of all factors were found to be highly significant (p<0.05) except P*T which was found to be highly significant (p<0.005) (Table 4.6). The m value was found to be increasing from 31 Pa s^n (450MPa/ /1.59 min/25°C) to 123 Pa s^n (618.2MPa/10 min/25°C) increasing linearly with increase in intensity of treatment. Ahmed et al. (2003) also reported that increase in pressure level increases the m value of egg yolk. It was also found that various phospholipids present in egg yolk are responsible for their viscosity and they also provide stability to egg white by ovalbumin-phospholipids complex (Nakamura et al., 1988).

Table 4.5: Flow behavior index (n) and consistency coefficient (m) for upward and downward curve and constant A, B for hold curve of egg yolk

No.	Pressure	Time	Temp.	up curve		Hold Curve		Down Curve	
	MPa	min	°C	n	m	A value	B	n	M
1	350(-1)	15 (1)	35 (1)	0.113	39	25	280	0.180	22.6
2	450(0)	10 (0)	25 (0)	0.108	49	451	806	0.210	23.3
3	450(0)	10 (0)	25 (0)	0.105	48	473	787	0.193	22.8
4	350(-1)	5(-1)	15 (-1)	0.107	32	4	348	0.687	11.1
5	550(1)	5(-1)	35 (1)	0.101	75	4	628	0.156	24.2
6	550(1)	15 (1)	15 (-1)	0.102	93	129	968	0.221	21.4
7	350(-1)	5 (-1)	35 (1)	0.112	36	36	683	0.306	12.7
8	450(0)	10 (0)	25 (0)	0.108	48	454	759	0.234	21.9
9	550(1)	15 (1)	35 (1)	0.104	96	0.21	40	0.164	25.6
10	550(1)	5 (-1)	15 (-1)	0.099	73	124	494	0.234	25.7
11	350(-1)	15 (1)	15 (-1)	0.113	32	4	689	0.501	17.0
12	450(0)	10 (0)	25 (0)	0.109	48	473	761	0.213	23.4
13	450(0)	10 (0)	8.2 (-1.68)	0.101	55	14	641	0.591	13.9
14	450(0)	10 (0)	25 (0)	0.106	49	491	708	0.239	23.6
15	450(0)	18.4(1.68)	25 (0)	0.106	49	66	511	0.146	26.0
16	618.2(1.68)	10 (0)	25(0)	0.097	123	17	396	0.129	33.5
17	450 (0)	10 (0)	41.8 (1.68)	0.104	62	0.36	312	0.169	19.9
18	281.8(-1.68)	10(0)	25 (0)	0.113	39	17	347	0.562	17.9
19	450(0)	1.59(-1.68)	25 (0)	0.101	31	45	579	0.314	18.7
20	450(0)	10 (0)	25 (0)	0.104	49	441	722	0.207	22.3

Table 4.6: ANOVA and regression coefficients of the second-order polynomial model for the response variables for egg yolk (upward, hold and downward flow curve)

		Flow Behavior Index			Consistency Coefficient		
	Source	Sum of Squares	DF	Prob > F	Sum of Squares	DF	Prob > F
	Block	4.36422E-05	2		8.69	2	
	Model	0.000389174	9	< 0.0001	11266	9	< 0.0001
Linear	**P**	3.2067E-004	1	< 0.0001	8424	1	< 0.0001
	t	3.769E-05	1	0.0001	405	1	< 0.0001
	T	1.45011E-05	1	0.0029	58.2	1	< 0.0001
Quadratic	**P^2**	5.35876E-09	1	0.9371	1844	1	< 0.0001
	t^2	3.761E-06	1	0.0631	131	1	< 0.0001
	T^2	8.676E-06	1	0.0113	159	1	< 0.0001
Interaction	**P*t**	2.8125E-07	1	0.5717	182	1	< 0.0001
	P*T	1.25E-09	1	0.9696*	4.5	1	< 0.0001
	t*T	4.35E-06	1	0.0490	1.58	1	0.0018
	Residual	6.470E-006	8		0.6	8	
	Lack of Fit	1.410E-006	5	0.96 *	0.54	5	0.094**
	R-Squared	0.98			0.99		
Hold Curve		Constant A			Constant B		
	Block	9656	2		40967	2	
	Model	782451	9	< 0.0001	938589	9	< 0.0001
Linear	**P**	2584	1	0.1203*	3299	1	0.0064
	T	45	1	0.8237*	6171	1	0.0010
	T	3497	1	0.0778	148197	1	< 0.0001
Quadratic	**P^2**	318725	1	< 0.0001	209836	1	< 0.0001
	t^2	263693	1	< 0.0001	50346	1	< 0.0001
	T^2	334667	1	< 0.0001	100342	1	< 0.0001
Interaction	**P*t**	22	1	0.8773*	330	1	0.2801*
	P*T	11367	1	0.0065*	64781	1	< 0.0001
	t*T	48	1	0.8194*	407679	1	< 0.0001
	Residual	6840	8		1966	8	
	Lack of Fit	5164	5	0.3251*	1692.15	5	0.1547*
	R-Squared	0.991			0.998		
Down Curve		Flow Behaviour Index			Consistency Coefficient		
	Block	1.847E-003	2		4.92	2	
	Model	0.51	9	< 0.0001	494.27	9	< 0.0001
Linear	**P**	0.19	1	< 0.0001	261.26	1	< 0.0001
	T	0.026	1	0.0001	45.80	1	< 0.0001
	T	0.18	1	< 0.0001	28.87	1	0.0005
Quadratic	**P^2**	0.025	1	0.0001	6.12	1	0.0308
	t^2	1.005E-005	1	0.8946*	4.12	1	0.0640*
	T^2	0.042	1	< 0.0001	86.65	1	< 0.0001
Interaction	**P*t**	0.012	1	0.0016	43.09	1	0.0001
	P*T	0.040	1	<0.0001	2.57	1	0.1287*
	t*T	8.080E-004	1	0.2549*	12.11	1	0.0062*
	Residual	4.297E-004	8		7.15	8	
	Lack of Fit	3.414E-003	5	<0.2602 *	4.99	5	<0.4190*NS*
	R-Squared	0.991			0.985		

* = Not significant, *P=pressure, t= time, T=temperature.

Hold curve: Results indicated that for constant A, model was highly significant (p<0.05), and even quadratic effects of pressure, time and temperature were significant (p<0.05); their interaction effect was found to be non-significant (Table 4.6). Lack of fit was found to be non-significant (0.96). Weltman A and B constant showed increase with increase in treatment intensity varying in between due to variation in process variables (Table 4.5). Increasing value of Weltman A constant signifies that there is increasing resistance to start up shear. Similarly on the other hand, increasing constant B value indicates increased rate of structure breakdown (Ramaswamy and Basak, 1992). For egg yolk, both A and B values increased with an increase in treatment intensity which indicated that the increasing treatment intensity helps in building up of startup viscosity and sensitivity to stress decay. Viscosity of EY samples remained higher throughout the study in comparison to EW and WLE indicating most prevailing effect on structure build up of EY.

Downward curve: The model was highly significant (p<0.05)) for n value. Similarly, linear effect of all process variables, quadratic effects of temperature and interaction effect of pressure and time were significant (p<0.05). Lack of fit was found to be non-significant which signifies correctness of model used. EY exhibited pseudo plastic behavior as depicted by n value <1 (Table 4.5). For EW, an increase in intensity of process resulted in an increase in n value.

For m value, the model, linear effect of all factors, quadratic effect of temperature and interaction effect of pressure*time were found to be highly significant (p<0.05) and R^2 value of 0.98 was found. In downward curve, their linear effect was significant (p<0.05) and interaction effect of pressure and temperature was also significant (p <0.05) (Table 4.6).

Egg yolk had higher viscosity in comparison to EW and WLE. Egg yolk was found to be turning from liquid to semi viscous liquid at pressure-temperature treatment (350 MPa/35°C/5min) with n value of 0.112 and afterwards it turned semi viscous liquid. The main reason for increase in thixotrophy of egg yolk after pressurization could be due to structural changes as it contains low and high density lipoproteins (Parkinson, 1977).

The low values of n value signifies that the egg yolk in upward and downward curve, show flow properties differing from that of the Newtonian behavior and like many other shear-thinning food products, they have a high viscosity at low shear rates which decreases dramatically as the shear is increased. It has been reported that a non-Newtonian behavior became important when the flow behavior index is less than 0.6 (Koocheki et al., 2008).

4.4.1.3 Effect of process variables on flow characteristics of whole liquid egg

It was found that all process variables played an important role in affecting all rheological variables. Similarly, it was evident that both flow behavior index (n) and consistency coefficient (m) were significantly affected by pressure, time and temperature (p<0.05). Regression model was significant for both responses (p<0.05). It was found that high pressure caused coagulation of whole liquid egg and they had different rheological behavior from control sample due to structural breakdown (Ahmed et al., 2003).

In a comparable study, it was concluded that even 400 MPa does not cause complete gelation of egg as caused by 80°C/30 min and it was dependent on protein content and –SH groups. Solubility remains stable at low protein content but with increase in protein content, solubility decreased. Amount of – SH groups increased considerably after pressure treatments (Van Camp and Huyghebaert, 1995).

In upward curve, shear stress of 0-100 s^{-1} was applied over time period of 5 min. Pseudo plastic behavior (n <1) was shown by WLE indicating structural breakdown. P values specified that linear and quadratic effect of all process variables, interaction effect of P*t, P*T and t*T had highly significant effect (p<0.05) on the n value. Lack of fit was found to be non significant (p<0.05) indicating high significance of model used (Tables 4.7 & 4.8).

For consistency coefficient (m value), linear (p<0.05), quadratic (p<0.05), interaction effect of P*t (p<0.05), P*T (p<0.05) and t*T (p<0.05) had significant effect

on m value. Lack of fit was found to be non-significant suggesting that this model is good for predicting response variable at different processing conditions.

In hold curve, constants A and B were calculated using Weltman's equation. These constant explain about effect of constant shearing on treated samples. In hold curve, Table 4.8 indicated that pressure had more significant effect (p<0.05) than that of temperature (p<0.05) and time (p<0.05) on the constant A value. Model was found to be significant (p<0.05) for constant B values. Linear, quadratic and interaction effect of all variables were found to have significant effect (p<0.05) on constant B except interaction effect of t*T (p<0.05).

Table 4.7: Flow behavior index (n) and consistency coefficient (m) for both upward and downward curve and constants A, B for hold curve of whole liquid egg

No.	Pressure	Time	Temp.	up curve		Hold Curve		Down Curve	
	MPa	min	°C	N	m	A value	B value	n	m
1	350(-1)	15 (1)	35 (1)	0.241	16	4	9	0.615	5.2
2	450(0)	10 (0)	25 (0)	0.209	26	17	75	0.545	2.4
3	450(0)	10 (0)	25 (0)	0.205	25	17	75	0.545	2.4
4	350(-1)	5(-1)	15 (-1)	0.367	1	2	8	0.705	0.1
5	550(1)	5(-1)	35 (1)	0.243	22	23	94	0.386	5.6
6	550(1)	15 (1)	15 (-1)	0.291	30	21	105	0.331	7.9
7	350(-1)	5 (-1)	35 (1)	0.301	2	2	11	0.607	3.3
8	450(0)	10 (0)	25 (0)	0.206	25	11	72	0.582	1.0
9	550(1)	15 (1)	35 (1)	0.156	41	17	138	0.142	7.8
10	550(1)	5 (-1)	15 (-1)	0.341	12	5	50	0.624	2.3
11	350(-1)	15 (1)	15 (-1)	0.312	3	1	4	0.799	0.1
12	450(0)	10 (0)	25 (0)	0.208	24	14	72	0.580	1.0
13	450(0)	10 (0)	8.2 (-1.68)	0.365	1	2	17	0.630	0.3
14	450(0)	10 (0)	25 (0)	0.206	24	14	71	0.550	0.6
15	450(0)	18.4(1.68)	25 (0)	0.207	28	11	53	0.386	4.4
16	618.2(1.68)	10 (0)	25(0)	0.236	37	26	159	0.313	6.3
17	450 (0)	10 (0)	41.8 (1.68)	0.215	22	7.92	54	0.333	5.3
18	281.8(-1.68)	10(0)	25 (0)	0.314	2	2	9	0.831	0.1
19	450(0)	1.59(-1.68)	25 (0)	0.311	2	2	12	0.574	0.4
20	450(0)	10 (0)	25 (0)	0.206	25	15	70	0.552	0.7

Table 4.8: ANOVA analysis and regression coefficient of second order polynomial model for response variables of up upward curve for whole liquid egg

		Flow Behaviour Index			Consistency Coefficient		
	Source	Sum of	D	Prob > F	Sum of	DF	Prob > F
	Block	9.57113E-05	2		24.3	2	
	Model	0.0736	9	< 0.0001	3031.1	9	< 0.0001
Linear	**P**	7.577 E-003	1	< 0.0001	1485.9	1	< 0.0001
	t	0.0134	1	< 0.0001	682.6	1	< 0.0001
	T	0.0283	1	< 0.0001	353.9	1	< 0.0001
Quadratic	**P^2**	0.00886	1	< 0.0001	35.9	1	0.0058
	t^2	0.00526	1	< 0.0001	159.3	1	< 0.0001
	T^2	0.013028	1	< 0.0001	289.8	1	< 0.0001
Interaction	**P*t**	6.84E-05	1	0.0188	60.8	1	0.0013
	P*T	0.001128	1	< 0.0001	9.1	1	0.0968
	t*T	0.000214	1	0.0008	17.6	1	0.0309
	Residual	6.35E-05	8	-	20.57	8	
	Lack of	5.74E-05	5	0.0926NSS	18.29	5	0.1129N
	R-Squared	0.999					0.993
Hold Curve		Constant A			Constant B		
	Block	93.1	2		99.7	2	
	Model	1118.0	9	< 0.0001	37818	9	< 0.0001
Linear	**P**	707.4	1	< 0.0001	27104	1	< 0.0001
	T	51.6	1	0.0010	1956	1	< 0.0001
	T	55.0	1	0.0008	1579	1	< 0.0001
Quadratic	**P^2**	4.004E -005	1	0.9966	322	1	< 0.0001
	t^2	102.4	1	0.0001	2658	1	< 0.0001
	T^2	154.9	1	< 0.0001	2240	1	< 0.0001
Interaction	**P*t**	11.6	1	0.0442	1392	1	< 0.0001
	P*T	15.2	1	0.0259	585	1	< 0.0001
	t*T	38.4	1	0.0025	7.9	1	0.0009
	Residual	16.4	8		2.4	8	
	Lack of	10.3	5	0.5318NS	1.6	5	0.4700
	R-Squared	0.985			0.999		
Down		Flow Behaviour Index			Consistency Coefficient		
	Block	0.00496	2		10.1	2	
	Model	0.546503	9	< 0.0001	126.6	9	< 0.0001
Linear	**P**	0.327247	1	< 0.0001	47.4	1	< 0.0001
	T	0.04127	1	< 0.0001	19.9	1	< 0.0001
	T	0.107051	1	< 0.0001	29.2	1	< 0.0001
Quadratic	**P^2**	0.001075	1	< 0.0001	11.8	1	< 0.0001
	t^2	0.008376	1	< 0.0001	5.696	1	< 0.0001
	T^2	0.007844	1	< 0.0001	8.653	1	< 0.0001
Interaction	**P*t**	0.050944	1	< 0.0001	4.517	1	< 0.0001
	P*T	0.002614	1	< 0.0001	3.266	1	< 0.0001
	t*T	0.000169	1	0.0007	0.287	1	< 0.0001
	Residual	4.7E-05	8		0.023	8	
	Lack of	4.05E-05	5	0.1540NS**	0.022	5	0.0585NS*
	R-Squared	0.999			0.998		

*NS = Non significant, *P=pressure, t= time, T=temperature.

In downward curve, effect of linear, quadratic and their interactions (p<0.05) except interaction of time*temperature (p<0.05) were found to have significantly impact on the n value (Table 4.8). For downward flow curve, consistency coefficient was found to have been significantly affected by linear, quadratic and interaction effects of all process variables (p<0.05).

Thus in downward curve, almost all process variables have equal effect in terms of influencing consistency coefficient. Thus high temperature and high holding time work in synergistic manner to increase flow behavior index in upward curve, whereas these follow reverse trend in downward curve.

Whole liquid egg showed different pattern of increasing from EW and EY. Viscosity of WLE was found to be increasing after pressure temperature treatment (450MPa/25°C/10 min) resulting in n value of 0.206. Higher pressure temperature treatments resulted in semi viscous liquids. High pressure and temperature causes unfolding of individual proteins in whole liquid egg which in turn governs the exposure of specific groups which causes change in rheology.

It has been found that high pressure and temperature caused changes in synergistic interactions of proteins in WLE that influenced rheological behavior of egg components. Howell and Lawrie (1984) proposed that the synergistic interactions between compatible small globular proteins were dependent on the degree of unfolding of the individual proteins in the mixture which governed optimum exposure of specific groups and thereby optimum interaction. The protein–protein interactions in the food protein aggregates are dependent on covalent, disulphide bonds. It also affects peptide backbone conformation as well as the microenvironment around the side chains changing interactions of individual and combined egg protein gels (Howrie and Lowrie, 1984; Li-Chan, 1996).

4.4.2 Optimization

The optimal conditions for flow properties of egg components by varying experimental ranges of pressure, time and temperature was done using optimization function of design expert software. Optimization was applied to pressure, time and

temperature levels of upward curve of egg components in order to get nearly same flow properties as that of pasteurized egg. Ponce et al. (1999) reported that the inactivation of *S. enteritidis* was obtained at pressure treatment of 450 MPa at 20°C for two cycles of 5 min (pasteurization condition). Based on this, a criterion was designed to use pressure, temperature and time combinations in such a manner that it should meet minimum requirements for pasteurization (450MPa/20°C/10 min).

Central point employed (450MPa/25°C/10min) in CCRD design were found to meet standards for pasteurization as per earlier literature (Ponce et al., 1999). Detailed results have been included in Table 4.9. 450MPa/25°C/10 min were taken as optimum conditions and m and n values of the upward curve of egg components after these treatments were taken as target values for optimization. Pressure treatment of several conditions were found with desirability 1, but the condition employing lowest pressure level for EW was 433MPa/14.6min/21.2°C which gave n and m value of 0.55 and 2.38Pa.s^n respectively.

Table 4.9: Optimization results of EW (upward curve) by desirability function.

Constraints	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Pressure	is in range	350	550	1	1	3
Time	is in range	5	15	1	1	3
Temp	is in range	15	35	1	1	3
FB index	is target = 0.55	0.184	0.7988	1	1	3
C. Coeff.	is target = 2.38	0.023	14.6	1	1	3
Number	Pressure	Time	Temp	F.B. Index	C. Coeff.	Desirability
1	479.77	12.00	15.33	0.55	2.38	1.00
2	432.74	14.53	21.17	0.55	2.38	1.00
3	464.72	12.54	17.30	0.55	2.38	1.00
4	442.42	13.78	20.05	0.55	2.38	1.00
5	451.74	13.19	18.93	0.55	2.38	1.00
6	472.15	12.25	16.34	0.55	2.38	1.00
7	441.43	13.85	20.17	0.55	2.38	1.00
8	458.51	12.83	18.10	0.55	2.38	1.00
9	437.80	14.96	19.86	0.55	2.53	0.99
10	469.17	5.00	28.70	0.53	2.38	0.97

FB Index=Flow behavior index, C. Coeff. = Consistency coefficient

Similarly, condition employing 443.89MPa/14.64min/19.37°C (Table 4.10) and 460.87MPa/10.92min/23.32°C (Table 4.11) were found optimum for egg yolk and whole liquid egg respectively. The desirability method is one of the most commonly used approach for optimization in industry. This method is based on theory that quality of process that has multiple quality parameters is unacceptable when even one of these parameters stays outside of desired range. In this study, desirability was found to be 1 in most of cases, ensuring that conditions employed in this study for optimization were highly precise.

Table 4.10: Optimization results of EY (upward curve) by desirability function

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Pressure	is in range	350	550	1	1	3
Time	is in range	5	15	1	1	3
Temp	is in range	15	35	1	1	3
FB index	is target = 0.108	0.097	0.1132	1	1	3
C. Coeff.	is target = 48.600	31.18	122.94	1	1	3

Number	Pressure	Time	Temp	F.B. Index	C. Coeff.	Desirability
1	443.89	14.64	19.37	0.108	48.604	1.00
2	432.40	9.25	34.62	0.108	48.605	1.00
3	444.29	13.55	21.83	0.108	48.599	1.00
4	444.01	14.32	20.05	0.108	48.602	1.00
5	439.28	10.46	30.90	0.108	48.600	1.00
6	444.31	13.46	22.06	0.108	48.599	1.00
7	444.12	14.07	20.60	0.108	48.602	1.00
8	433.00	9.33	34.36	0.108	48.599	1.00
9	439.78	10.58	30.53	0.108	48.604	1.00

FB Index=Flow behavior index, C. Coeff. = Consistency coefficient

Table 4.11: Optimization results of WLE (upward curve) by desirability function

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Pressure	is in range	350	550	1	1	3
Time	is in range	5	15	1	1	3
Temp	is in range	15	35	1	1	3
FB index	is target = 0.208	0.156	0.3671	1	1	3
C. Coeff	is target = 26.30	1.252	41.182	1	1	3

Number	Pressure	Time	Temp	F.B. Index	C. Coeff.	Desirability
1	460.87	10.92	23.32	0.208	26.299	1.00
2	465.86	10.29	23.77	0.208	26.300	1.00
3	463.03	10.63	23.51	0.208	26.300	1.00
4	466.88	10.17	23.87	0.208	26.301	1.00
5	506.22	7.42	29.07	0.208	26.299	1.00
6	452.91	12.23	22.75	0.208	26.299	1.00
7	515.64	7.12	30.89	0.208	26.300	1.00
8	525.67	7.00	33.48	0.208	26.300	1.00
9	444.58	14.51	22.75	0.208	26.300	1.00
10	387.14	12.75	33.82	0.208	20.872	0.89

FB Index=Flow behavior index, C. Coeff. = Consistency coefficient

4.5 Conclusions

Rheological properties of egg components (WLE, EW and EY) were studied as a function of time, temperature and pressure. All of the egg components were showing shear thinning behavior. Whole liquid egg containing both the components of egg yolk and egg white behaved differently and showed an initial shear thinning followed by sudden gain in viscosity exhibiting thixotropy. At a higher pressure range, (550 - 618 MPa) viscosity of egg yolk increased to such a great extent that the yolk was not able to flow any longer. At such high pressure range, yolk was observed to be a highly viscous gel and that should be subjected to textural analysis. Further work on pressure-time-temperature combinations used to analyze highly viscous gels is required before implementing HPP for commercial application. Back extrusion could be a possible technique to further explore in detail the changes that occurred due to HPP at higher levels of pressures.

PREFACE TO CHAPTER 5

In the previous chapter, effect of high pressure processing on rheology of various egg components was studied and it was found that increase in pressure level increases the viscosity of egg components to a point where they can't be evaluated using rheology. Increase in viscosity is due to denaturation and aggregation of egg proteins. Back extrusion is used to evaluate the flow properties of viscous fluids which have the consistency of a paste.

In this chapter, partially denatured egg components were evaluated using back extrusion technology. Similarly, back extrusion and rheology were compared for egg components which were in flowing state. This will give us background information about comparative usage of these methods for analysis of liquid (apparent viscosity) and semi-viscous (viscosity index) liquids. This comparative analysis will also allow us to understand the basis of changes occurring in the pressure treated egg components.

Part of this work has been presented at the Northeast Agricultural and Biological Engineering Conference, Vermont, USA in 2011. One publication has been prepared for submission from this chapter:

Singh A and Ramaswamy HS (2012) Effect of high pressure processing on back extrusion properties of various egg components (Prepared for Submission).

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy.

CHAPTER 5

EFFECT OF HIGH PRESSURE PROCESSING ON BACK EXTRUSION PROPERTIES OF VARIOUS EGG COMPONENTS

5.1 Abstract

Egg is well known for its nutritional and functional qualities as a product itself and as being an ingredient in other food preparations. A central composite design with three independent variables namely high pressure level (350-550MPa), pressure treatment time (10-20 min) and temperature (15-35°C) was used to study their effects on back extrusion properties (viscosity index (η) and apparent elasticity (Ea)) of various egg components (egg white, egg yolk and whole liquid egg). This information can also be used to build knowledge gap between liquid rheology and solid gel rheology (Texture Profile analysis). A second-order polynomial model was developed using multiple linear regression analysis for each response (η and Ea). Viscosity index and apparent elasticity of various egg components were found to be significantly increasing (p <0.05) with increase in treatment intensity. In a similar way, relationship between apparent viscosity (rheological) and viscosity index (textural properties) was established to correlate the behavior of various egg components. It can be used to predict the behavior of their respective products during consumption. They showed positive correlation for all egg components (egg white, egg yolk and whole liquid egg) as it was found to be r =0.96, 0.99 and 0.99.

5.2 Introduction

Proteins are used as functional ingredients in a variety of foods preparations due to their foaming, gelling and functional properties. It is a highly nutritional food component, which supplies utmost level of nutrition required for growth and maintenance in the human body. Proteins are principally used as fluid suspensions in foods. These suspensions exhibit a wide range of flow behaviors from time independent Newtonian to

time dependant Non Newtonian characteristics depending on their origin, composition and structure (Rao, 1986). Proteins cover a broad range of rheological behaviors (pseudoplastic, shear thickening, thixotropic and viscoelastic) due to their complex structure and composition. Rheological properties of proteins have a significant impact on the overall acceptability of food system. Rheological characteristics can also act as a support index to establish the optimized values for transportation of liquid or semi solid foods throughout the processing line, during pasteurization or the design of the packaging units such as tetra pack, containers etc. Hence, the analytical study of rheology became an attractive and essential area of research for scientists.

Egg is an economical and good quality source of proteins and it has exceptional standing for providing highest level of nutrition for the human body. It has been pasteurized mostly using thermal processing techniques which may alter its coagulating, foaming and emulsifying properties. In order to extend the shelf life of eggs and to achieve desirable structure modifications, many novel food processing techniques have been explored by researchers such as ultra pasteurization in combination with aseptic packaging, high pulsed electric field (Belloso et al., 1997), while high hydrostatic pressure treatment (Ahmed et al., 2003; Anton et al., 2001; Ngarize et al., 2005) has shown that it can be used without much alteration in the quality characteristics.

HPP can induce changes in protein conformation which in turn causes modifications of functional and rheological properties (Lametti et al., 1998, Lametti et al., 1999 and Knorr et al., 1992). This protein denaturation can lead to aggregation or gelation depending on the number of factors related to protein system (nature and composition of proteins), environmental conditions (pH and ionic strength) and HP treatment conditions (pressure level, processing time and temperature) (Messens et al., 1997). Knowledge of the rheological properties of food products is essential for the product development, quality control, sensory evaluation and design and evaluation of the process equipment (Ahmed et al., 2003).

Rheological properties of HPP treated whole egg and its components have been studied widely in liquid and completely gelled conditions (Hayashi et al., 1989; Hosseini-nia et al., 2002). Natural egg components without HP treatment have been reported to

exhibit shear thinning pseudoplastic behavior (Atilgan and Unluturk, 2008) at certain temperature ranges. Similarly, effect of high pressure processing on coagulation of whole egg was studied and it was reported that structural breakdown is responsible for change in rheological properties (Lee et al., 1999). Moreover, high temperature and pressure can cause changes in structure and protein confirmation of egg leading to gelation of egg samples (Okamoto et al., 1990). Any changes in the structure of protein can affect the rheological behavior of the food systems to a great extent; hence it would be exciting to explore the flow of HP treated egg.

Conventional rheology or texture measurements cannot be used to accurately evaluate partially coagulated egg samples which are neither in liquid nor gel. In order to study these rheological characteristics, back extrusion properties can be used (Hickson et al., 1982). Back extrusion can be used as a bridge to link the information gap between liquid and gelled egg samples so as to evaluate intermediate semi solid samples. In back extrusion evaluation, plunger is forced downwards into the sample present in annular tube and sample flows out of the tube and gives deformation curve (Dolan et al., 1989). Back extrusion technique is very simple and requires only a texture meter, which is easy to operate and readily available. Food consumers have distinct expectations towards liquid, semi solid and gel food properties.To build up in between gap and meet these expectations, it is very important to critically analyze and define the relationship between the flow properties and back extrusion especially in the areas of product development. This technique can be used for automated sampling and rheological evaluation, thus it has great prospective in food industry.

In order to test the effect of high pressure processing on the rheological parameters of egg, systematic move toward reckoning overall rheological back extrusion properties was prepared by connecting liquid, semi-solid and gel rheology. The overall objective of this study was to evaluate the changes in back extrusion properties of egg components and development of relationship between back extrusion and rheological properties as affected by application of HP treatments at various time, temp and pressure combinations using response surface methodology.

5.3 Materials and methods

Raw liquid eggs were purchased from local market. Only large size eggs were taken for all experiments and were sorted to exclude any cracked or inferior quality eggs. The eggs were broken down and mixed well to obtain a sample of whole liquid egg (WLE). For the egg yolk (EY) and egg white (EW), eggs were carefully broken from the top and egg white was allowed to pour slowly leaving the yolk behind. Yolk was cleaned with the filter paper so as to remove any residue of egg white. Separated egg yolk and egg white were individually packed in flexible polyethylene pouches designed specifically for processing treatments.

5.3.1 High hydrostatic pressure treatment

HP treatments were carried out in an Isostatic press (Model ACIP 9000/1.7/8.5VB, ACB Corp, Nantes, France) with a cylindrical pressure chamber (8.5 cm diameter and 30 cm high). A high temperature circulating water bath (VWR model 1197) was connected to HP chamber in order to control the temperature during the processing. The pressurization medium used was pure water. Pressurization and depressurization rate of 4.4 MPa/s and 26 MPa/s respectively, were used during high pressure treatments. Samples (WLE, EW and EY) were packed in 2 oz. polyethylene bags (Whirl Pak$^{(R)}$, USA. These samples were submerged in water at a desired temp for the pressurized treatment for a specific length of time. Pressure treatment time suggested by CCRD design in the study did not consider the pressure build up or releasing time. The pressure-treated pouches were immediately transferred to a refrigerator (4°C) followed by rheological analysis. The overall treatment cycle consisted of 3 phases-pressurization, pressure holding and depressurization. Duplicate measurements were carried out for each pressure and temperature combinations for all the samples.

5.3.2 CCRD design

Experiments were designed using central composite rotatable design (CCRD) to model effect of three variables on back extrusion properties of egg components in 20

experiments, where X_1= pressure (350-550 MPa), X_2= treatment time (5-25min), &X_3= temperature (15-35°C).

RSM was applied to the experimental data using a commercial statistical package, Design-Expert version 6.01 (Stat ease Inc, Minneapolis, USA). RSM was used as it is one of worldwide accepted optimization technique due to its comprehensive efficiency and simplicity (Arteaga et al., 1994). Response functions calculated were η (Y_1) and apparent elasticity (Y_2). Quadratic polynomial regression model was used for correlating these values to their coded variables (x_i, i = 1, 2, and 3).

$$Y=b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \qquad (5.1)$$

where b_0 (constant term), b_1, b_2 and b_3 (linear effects), b_{11}, b_{22} and b_{33} (quadratic effects), and b_{12}, b_{13} and b_{23} (interaction effects) represents the coefficients of the polynomial model. ANOVA analysis was used for evaluating statistical significance of terms in regression. R^2 value was used to evaluate efficacy of model (Montgomery and Myers, 2002).

5.3.3 Back-extrusion rheology

Samples (WLE, EY, EW) treated with HPP were evaluated for changes in flow properties using back extrusion rheology approach (Hickson et al., 1982) by employing TA-TX plus texture analyzer (Texture technologies, Scarsdale, NY, USA). Rheological parameters and force required to penetrate gel (initial penetration force) were measured using force deformation curve. In this study, back extrusion was used to calculate viscosity index and apparent elasticity. Viscosity index was calculated using counter flow back extrusion model (Equation 5.2). From this model, elastic measurements were not ideal values as stress and strain values were not absolute and these apparent stress and strain values were calculated using equation 5.3 (Hickson et al., 1982). TA.TX2 texture meter was used for back extrusion of different high pressure treated egg components. The technique involves intruding a TA.TX ½ inch probe into the cylindrical container containing the egg sample. By lowering a plunger, the egg samples were forced upwards between the wall and the plunger. The necessary back extrusion force was delivered and

measured by a texture test system at constant plunger velocity. The measurements were executed and repeated when big deviations occurred because of inclusion of air in the samples. Pre test speed of 0.1 mm/s, test speed of 1 mm/s and post test speed of 1 mm/s was used for calculating peak force. Apparent viscosity and apparent elasticity were calculated by using the following formulae:

$$Viscosity\ Index\ (\eta) = \left(\frac{1}{2\prod Vp}\ \frac{Fp}{Lp}\ \ (K)^2\ ln\frac{1}{k}\ \left[1 + \frac{\alpha}{\ln k}\right]\right. \tag{5.2}$$

$$Apparent\ elasticity\ (E_{a)=\frac{\sigma}{\epsilon}}\ 2\prod \mathrm{V_p} \tag{5.3}$$

This test has been well described by Osorio and Steffe, (1987) and Morgan et al., (1982). The technology has been used to determine rheological behavior of egg by Hickson et al., 1982. Back extrusion has been used to determine the rheological behavior of tomato concentrates (Alviar and Reid, 1990) and mustard slurry (Brusewitz and Yu, 1996).

5.3.4 Comparison of back extrusion and rheological properties of egg components

The rheological behavior of egg components was characterized in controlled stress rheometer (AR 2000, TA Instruments, New Castle, DE, USA) with attached computer software (Rheology Advantage Data Analysis Program, TA). The egg components were sheared at a constant low shear rate (10 s^{-1}) for 300 s using parallel plate geometry (60mm geometry, 1mm gap). Apparent viscosity from rheological behavior was calculated using shear stress/shear rate data obtained as described in equation 5.4 (Rao, 1986).

$$\eta_a X = \frac{\sigma X}{\gamma X} \tag{5.4}$$

Here, σX is shear stress corresponding to a shear rate of γX.

On other hand, apparent viscosity was calculated with back extrusion technology using eq. 5.2. They were compared using regression values and correlation coefficient.

5.4 Results and discussions

Food products have tendency to exhibit viscosity, elasticity and rigidity depending upon type of protein, concentration and processing treatment used (Harper et al., 1978). Back extrusion technology was used to explore the effect of these factors on food gels.

5.4.1 Effect of processing treatments on egg components

Viscosity index (η) and apparent elasticity (Ea) of EW were calculated as function of pressure, treatment time and temperature (Table 5.1). Viscosity index is a measure used to evaluate change in viscosity of food sample. In similar way, apparent elasticity is the elastic limit of materials that do not have a significant straight line portion on stress/strain graph. It is used to measure elasticity of food products and gels. In this study, processing treatments at higher pressure were found to cause significant (p <0.05) increase in viscosity index and apparent elasticity of all egg components. For EW, mean value of viscosity index and apparent elasticity ranged from 214.9 - 472.9 poise and 18.8 - 49.81N/cm^2 respectively. On other hand, viscosity index and apparent elasticity varied from 671.88- 1737.81 poise and 16.72 - 433.96 N/cm^2 respectively in case of EY. Similar trends were observed in WLE as it ranged from 21.14 - 164.02 poise and 13.01 - 52.98 N/cm^2. These results specify broad range of responses varying as according to processing treatments. In other words, back extrusion properties were quite sensitive to the process variables justifying the need for the study and evaluating the optimal processing conditions required for egg formulations.

5.4.2 Data analysis and model fitting

Data on apparent viscosity and apparent elasticity was evaluated by CCRD experimental design. Back extrusion and apparent elasticity of all egg components was showing sensitivity to process variables as evident from the results in Table 5.1.

Table 5.1: Experimental design of process with actual variables and values of viscosity index and apparent elasticity for all egg components (EW, EY and WLE)

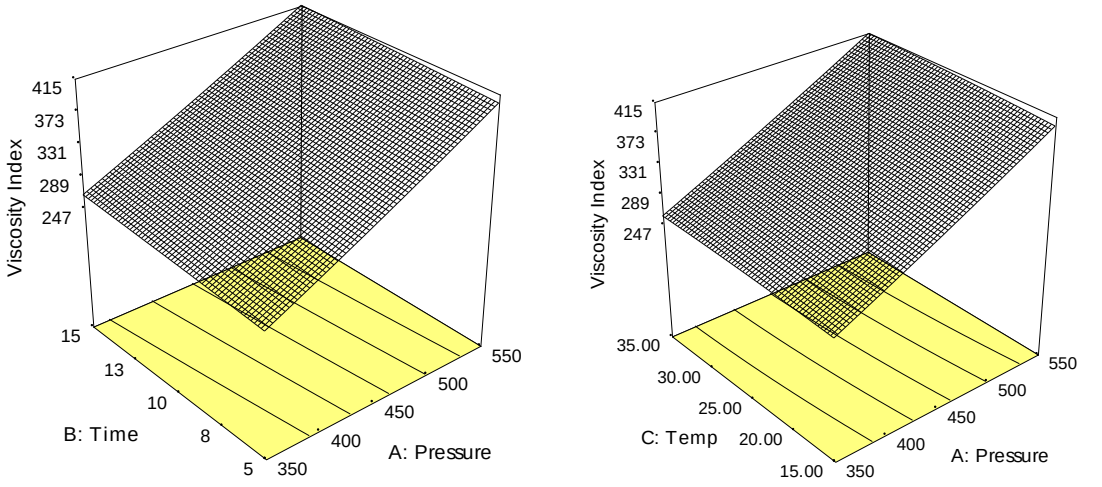
#	Pres	Time	Temp	EW		EY		WLE	
	MPa	min	°C	V.I.(η)	A.E.(Ea)	V.I.(η)	A.E.(Ea)	V.I.(η)	A.E.(Ea)
1	350	15	35	267.63	22.37	890.15	291.85	29.61	15.76
2	450	10	25	327.43	16.72	1737.81	304.38	28.40	16.72
3	450	10	25	328.05	16.72	1695.99	286.24	28.97	16.72
4	350	5	15	241.25	18.79	1317.07	339.95	24.40	15.10
5	550	5	35	401.87	37.60	1303.73	344.52	106.35	36.57
6	550	15	15	402.43	29.68	1093.99	202.56	104.09	36.68
7	350	5	35	242.17	13.21	853.20	281.15	24.62	15.27
8	450	10	25	327.27	16.04	1701.96	348.25	26.81	16.14
9	550	15	35	416.99	40.92	1224.28	392.64	122.36	42.87
10	550	5	15	395.01	35.68	785.13	249.90	94.66	35.83
11	350	15	15	252.25	15.03	1273.17	281.64	21.14	15.03
12	450	10	25	327.69	16.03	1704.04	297.17	26.35	13.01
13	450	10	8.2	323.85	25.21	878.22	371.64	59.39	32.26
14	450	10	25	337.20	16.57	1531.19	282.65	27.06	16.41
15	450	18.4	25	347.37	16.72	1507.16	16.72	39.57	16.72
16	618.2	10	25	472.98	49.81	930.95	301.74	164.02	52.98
17	450	10	41.8	340.75	31.59	671.88	433.96	76.52	28.66
18	281.8	10	25	214.89	18.87	795.56	331.33	21.84	14.16
19	450	1.6	25	324.34	15.45	1287.02	16.72	29.16	16.72
20	450	10	25	337.22	16.81	1657.77	292.87	28.97	16.81

The sum of squares of the sequential model was analyzed for the corresponding fitting of the explanatory models and the variation of the η and Ea. Regression analysis and ANOVA were used for developing and evaluating the statistical significance of the terms. The coefficient of determination (R^2) is the proportion of variation in the response attributed to the model rather than to random error (Little and Hills, 1978). Lower values of R^2 indicate that model used is not appropriate to explain the relation between variables. The R^2 values for response variables (η and Ea) was found to be >0.95 in all egg components ismplicating that the regression models were suitable to correlate the experimental variations.

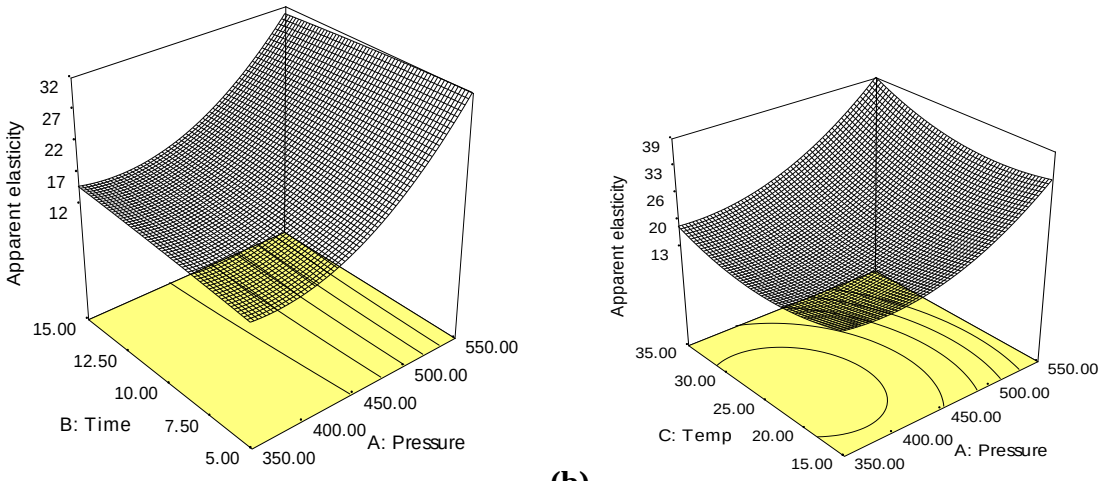
5.4.2.1 Egg white: The three dimensional response plots of viscosity index as function of pressure, time and temperature are presented in Figure 5.1. These 3D plots suggest that the viscosity index of egg white was found to be increasing significantly ($p < 0.05$) with increase in pressure level used but treatment time and temperature used were not significant ($p > 0.05$) in terms of affecting viscosity index. The second order polynomial surface model was fitted to each response i.e. "Y" as shown in Equation 5.1. Quadratic model was signified by program to give paramount performance for η. Similarly, ANOVA analysis indicated that linear, quadratic and interaction effect of all factors were found to be highly significant ($p < 0.05$). Furthermore, lack of fit was found to be non-significant which indicated that model used has high significance. Sequential sum of squares was used for judging model fitting and variation in values of process responses (Table 5.2).

Based on the sum of squares, the importance of the independent variables on η of egg white could be ranked in the following order: Pressure level > Treatment time> Temperature. The main rationale behind the increasing viscosity following high pressure is that it can expose and break disulfide bonds responsible for protein structure resulting in improvement of functional attributes (Hosseini-nia et al., 2002). The breaking of disulfide bonds can also improve functional properties in significant manner (Doir et al., 1997).

It was found that the HPP cause formation of finite size soluble egg white aggregates, which were constructed mainly from disulfide cross-links and β-sheet hydrophobic forces and they play an important role in improvement of foaming properties (Mine et al., 1997). Lyzozyme present in egg white may interact with ovomucin and play a role in interaction with other proteins during foaming and gelation (Mine et al., 1997).Thus high pressure can cause increase in protein-protein interaction which in turn increases viscosity and can cause gelation (Anson and Mirsky, 1931). Figure 5.1a shows effect of processing factors on EW, as with increase in pressure throughout the entire range, it showed significant increase in viscosity index (η) value, whereas it remained constant with increase in time and temperature level. The increase in viscosity index and consistency was mainly due to gelation of egg protein systems.



(a)



(b)

Figure 5.1: Three-dimensional (3D) response surface plots showing the effect of the variable on the response of EW: (a) the effect of pressure, time and temperature on viscosity index; (b) the effect of pressure, time and temperature on apparent elasticity

Table 5.2: Sequential model sum of squares for viscosity index and apparent elasticity (Egg white)

	Egg White	Viscosity Index			Apparent elasticity		
	Source	Sum of Squares	DF	Prob > F	Sum of Squares	DF	Prob > F
	Block	467.5133	2		4.06	2	
	Model	81527.97	9	< 0.0001	2074	9	< 0.0001
Linear	P	80276.72	1	< 0.0001	1171	1	< 0.0001
	T	699.6594	1	< 0.0001	1.729	1	< 0.0001
	T	320.4339	1	< 0.0001	48.07	1	< 0.0001
Quadratic	P^2	76.56458	1	< 0.0001	565.36	1	< 0.0001
	t^2	4.373976	1	0.0094	0.506	1	0.0004
	T^2	47.07431	1	< 0.0001	249.86	1	< 0.0001
Interaction	P*t	24.20318	1	< 0.0001	8.164	1	< 0.0001
	P*T	3.279914	1	0.0186	16.29	1	< 0.0001
	t*T	61.44014	1	< 0.0001	61.8	1	< 0.0001
	Residual	3.029158	8		0.121	8	
	Lack of Fit	2.74795	5	0.0881NS	0.0919	5	0.3226N
	R-Squared	0.9999			0.99990		

This fact was also exhibited in study which exhibited progressive decrease in denaturation enthalpy with increase in pressure (Aguilar et al., 2007). Temperature and high pressure were found to be responsible for causing coagulation of egg white which in turn results in progressively increasing viscosity (Ahmed et al., 2003). But it was found that thermal process causes more textural changes in comparison to pressure. The energy generation from high pressure is only 2 kcal /mol per 10^5 bars and it could not completely disrupt those bonds but on the other hand thermal energy could provide the sufficient energy to cleavage those bonds (Hayakawa et al., 1992).

On other hand for apparent elasticity data, Table 5.1 accentuates the deformation aspect of apparent elasticity modulus. It was found that apparent elasticity exhibited significant ($p < 0.05$) increase with increase in pressure level used. 3D response plots show that apparent elasticity exhibited linear increase with increase in pressure level as compared to other treatment factors which caused marginal increase in Ea. ANOVA analysis exhibited that linear, quadratic and interaction effects of all factors showed high significance ($p <0.05$) except quadratic effect of time ($p >0.05$).

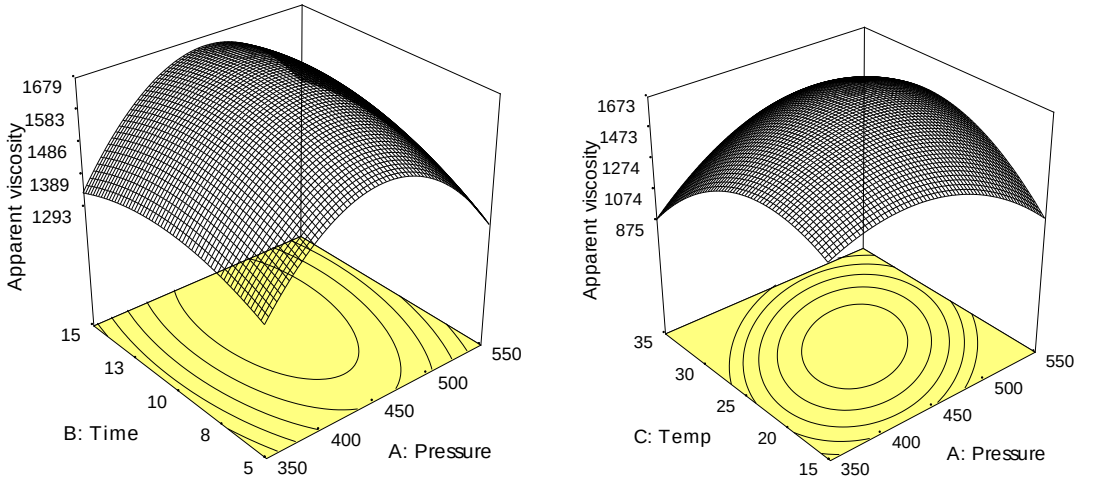
Lack of fit was found to be significant. High efficiency of data employed was shown by high R^2 values (Table 5.2). Apparent elasticity exhibited constant behavior with increase in initial phase, but afterwards it was followed by sharp increase with increase in pressure level (Figure 5.1b). This shows that pressure plays most important role followed by time and temperature in effecting η and Ea of EW. This study was in agreement with previous study in which apparent elasticity of egg white was found to be rising by increasing heating time and temperature (Hickson et al., 1982).

Following model equations (Equations 5.5 and 5.6) were developed for η and Ea as function of Pressure (P in MPa), temperature (T in °C) and treatment time (t in min). CCRD designs are statistics based experimental optimization models, thus relying on carrying smallest number of experiments. Therefore, it is necessary to develop response surface model equations for EW. These model equations can be used to generate η under different experimental conditions. The R^2 values (>0.95) were found to be highly significant for η in all egg components. These results indicate that polynomial models were in agreement with experimental data.

$$\text{Viscosity Index} = -3558+18.210*P+57.01679*t+64.3*T-0.02583*P^2-2.78*t^2 - 2.89*T^2+0.059*P*t+0.19*P*T-0.768*t*T \quad (R^2= 0.99) \qquad (5.5)$$

$$\text{Apparent elasticity} = 1197-2.79*P+54.20*t-44.47*T+1.77E-003*P^2-3.530*t^2 +0.482*T^2+0.0120*P*t+0.0416*P*T+0.41*t*T \quad (R^2 =0.99) \qquad (5.6)$$

5.4.2.2 Egg yolk: Viscosity index (η) of egg yolk varied significantly ($p < 0.05$) with increase in treatment intensity. Three dimensional graphs show that pressure and temperature caused the η to increase followed by decrease towards end (Figure 5.2). On the other hand, increase in treatment time caused minor increase in η of EY. Graphs illustrated that EY reached the peak viscosity at around pressure (450MPa) and temperature (25°C) and then it started decreasing toward peak of pressure (550 MPa) and temperature (35°C) treatment (Figure 5.2).



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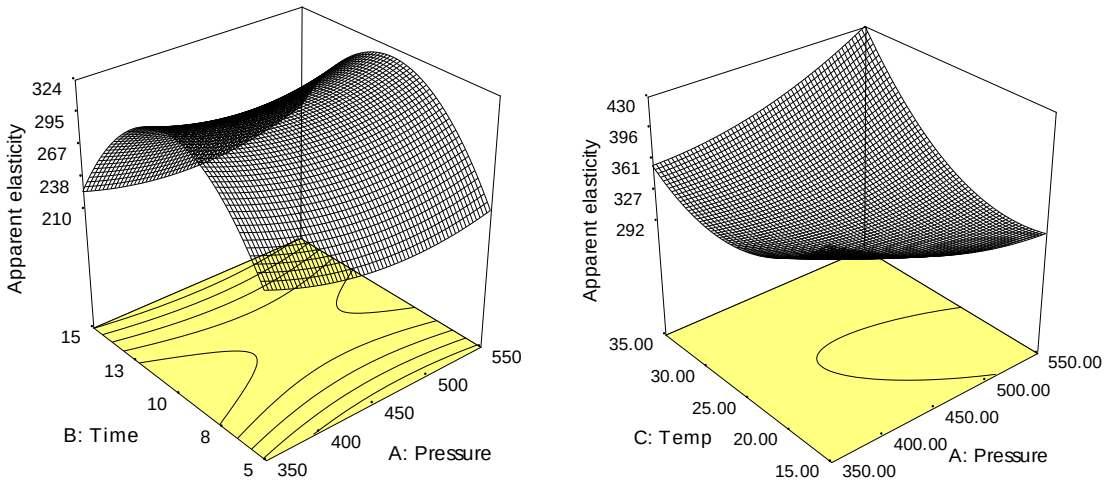


(b)

Figure 5.2: Three-dimensional (3D) response surface plots showing the effect of the variable on the EY: (a) the effect of pressure, time and temperature on viscosity index; (b) the effect of pressure, time and temperature on apparent elasticity

Table 5.3 eindicates that the model was found to be highly significant (p <0.05). It suggests that linear effect of time and temperature (p <0.05), quadratic effect of pressure and temperature (p <0.05) and interaction effect of Press*Temp had significant affect on the η. Lack of fit was found to be non significant. Based on the sum of squares, the importance of the independent variables on viscosity index could be ranked in the following order: Time > temperature > pressure. It seems that interaction effect of all factors have positive influence on η of egg yolk. In a corresponding study, It was found that increasing intensity of pressure increases viscosity of egg yolk emulsion prepared with 5.5% protein, the viscosity increased from 17.7 mPa.s for non-treated to 22.7 mPa.s at 200 MPa and 27.0 mPa.s at 500 MPa (Anton et al., 2001).

Table 5.3: **Sequential model sum of squares for viscosity index and apparent elasticity (Egg yolk)**

	V.I. Source	Sum of Squares	DF	Prob > F	Sum of Squares	Ea DF	Prob > F
	Block	115883	2		10589	2	
	Model	2331348	9	< 0.0001	192697	9	< 0.0001
linear	P	6645	1	0.2081	219	1	0.5032
	T	25721	1	0.0273	160	1	0.5654
	T	21752	1	0.0382	8510	1	0.0024
Quadratic	P^2	961334	1	< 0.0001	4526	1	0.0129
	t^2	69835	1	0.0022	112204	1	< 0.0001
	T^2	1207407	1	< 0.0001	33491	1	< 0.0001
Interaction	P*t	6984	1	0.1979	293	1	0.4415
	P*T	279667	1	< 0.0001	13886	1	0.0005
	t*T	11818	1	0.1052	3382	1	0.0250
	Residual	28342	8		3572	8	
	Lack of Fit	19455	5	0.4378	2050	5	0.6113
	R-Squared	0.987			R-Squared	0.981	

The possible cause for increase in consistency is possibly due to Low density lipoproteins (LDL) denaturation and disruption. The aggregations interactions occurring under these conditions are mostly hydrophobic. High pressure process can give a thickening effect due to increase in viscosity depending upon size of aggregates or 3 D structures (Mine et al., 1997).

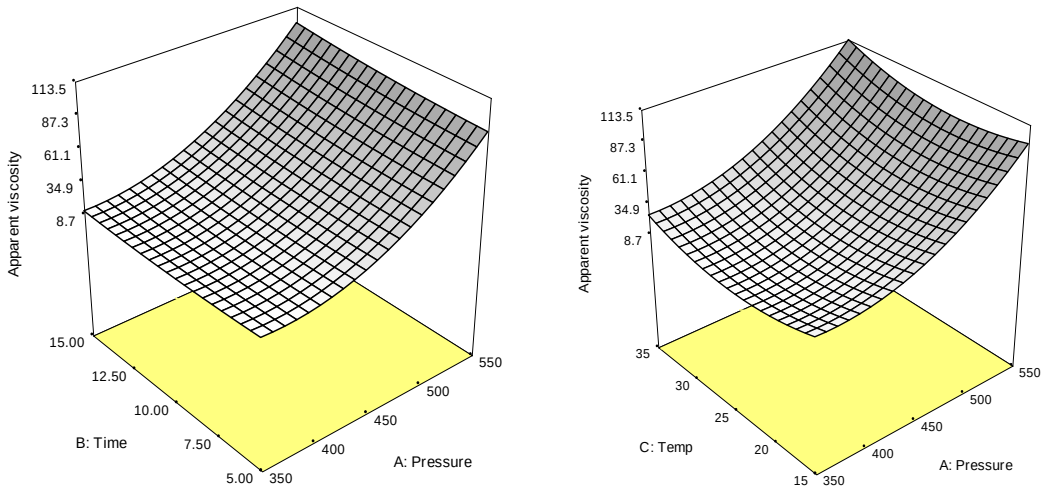
For apparent elasticity of egg yolk, it was observed that linear term of temperature, quadratic terms of all process variables and interaction effect of pressure*temp and time*temp had a significant effect (p <0.05) (Table 5.3). Treatment time caused significant (p <0.05) increase in apparent elasticity followed by decrease up to original values. Pressure and temperature were found to cause insignificant changes in apparent elasticity (Figure 5.2b).

It has been already reported that increase in treatment intensity causes increase in elasticity of protein suspensions (Harper et al., 1978). The viscosity of egg yolk rises with increase in temperature as it is sensible to heat treatment due to LDL which is a constituent responsible for increasing viscosity of egg yolk (Saari et al., 1964). Following polynomial model equations 5.7 and 5.8 were developed for η and apparent elasticity as function of pressure, temperature and time.

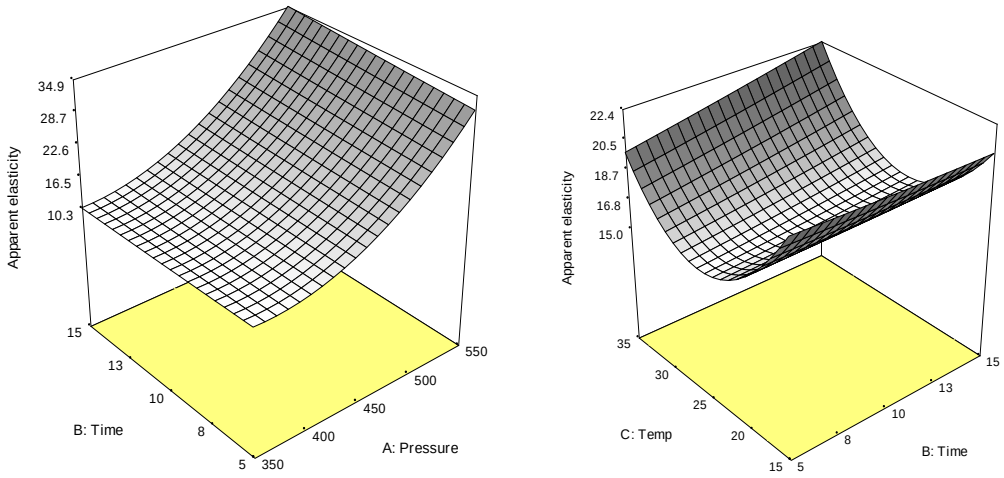
$$\text{Viscosity index} = -3558.149+18.209* P+57.01679v*t+64.34106* T-0.025838*P^2-2.78559t^2-2.89565*T^2+0.059092* P*t + 0.18697*P*T-0.76868*t* \quad (R^2 =0.97) \quad \textbf{(5.7)}$$

$$\text{Apparent elasticity} = 1197 -2.80*P+54.207*t- 44.47* T+1.77277E-003*P^2-3.53087 t^2+0.482*T +0.012099*P*t+0.041662*P* T+0.41119*t*T \quad (R^2=0.96) \quad \textbf{(5.8)}$$

5.4.2.3 Whole liquid egg: Analysis of WLE illustrated divergent behavior from EW and EY. Pressure was found to be a more significant factor (p <0.05) than treatment time and temperature (Figure 5.3). Increase in pressure level caused increase in η. The possible reason for different pattern followed by WLE than EW and EY could be due to fact that WLE contains EW and EY in different proportions and they were affected in diverse manner by processing treatments (Harms, 1993). Higher fat content (26-30%) in EY could be the possible reason of different behavior of EY than EW and WLE. It gave an observation similar to that of cream cheese. At higher pressure level (>450MPa), there was visible precipitation and coagulation of WLE. The coagulation was accountable for causing increase in its viscosity (Rao, 1977).



(a)



(b)

Figure 5.3: Three-dimensional (3D) response surface plots showing the effect of the variable on the response of WLE: (a) the effect of pressure, time and temperature on viscosity index; (b) the effect of pressure, time and temperature on apparent elasticity

The linear, quadratic and interaction effect of all factors were highly significant (p <0.05) (Table 5.4). Lack of fit was found to be non-significant which indicated that appropriate model has been used. The interaction effect of different variables on the responses can be depicted from three dimensional plots (Figure 5.3). Pressure caused significant increase in η (p <0.05) whereas time and temperature played a non-significant role (p <0.05).

Table 5.4: Sequential model sum of squares for viscosity index and apparent elasticity (Whole liquid egg)

		Viscosity Index			Apparent elasticity		
	Source	Sum of Squares	DF	Prob > F	Sum of Squares	DF	Prob > F
	Block	37.15	2		8.998	2	
	Model	33578	9	< 0.0001	2606	9	< 0.0001
Linear	P	23523	1	< 0.0001	1784	1	< 0.0001
	T	146.17	1	< 0.0001	4.205	1	0.3177
	T	333.01	1	< 0.0001	0.231	1	0.8092
Quadratic	P^2	7433	1	< 0.0001	519.92	1	< 0.0001
	t^2	58.29	1	0.0002	0.0393	1	0.9205
	T^2	2778	1	< 0.0001	346.94	1	< 0.0001
Interaction	P*t	70.29	1	0.0001	5.659	1	0.2514
	P*T	56.51	1	0.0002	4.535	1	0.3006
	t*T	27.47	1	0.0022	4.523	1	0.3012
	Residual	11.174	8		29.624	8	
	Lack of Fit	9.0882	5	0.2294	24.63	5	0.2004
	R-Squared	0.999			R-Squared	0.987	

In case of apparent elasticity, linear effects of pressure and quadratic effect of pressure and temperature were found to be highly significant (P>0.005). Model was found to be highly indicated as represented by good fit. Figure 5.3b shows that Ea was found to be increasing at linear rate with increase in pressure. In similar way, it was found that increase in temperature causes decrease in Ea followed by bouncing back to original value. On other hand, treatment time proved to be a non-significant factor. High pressure favors the denaturation process which has opposite effect on aggregation of globular proteins as at high net charge denaturation (Protein-solvent interaction) is favored over that of aggregation of protein-protein interaction). Optimized processing

conditions can result in aggregation that results in gel network with certain degree of order.

Aggregation take place more slowly than denaturation thus a denatured protein molecule has time to orient itself before aggregation which results in gels which are higher in elasticity and lower in opacity (Hermansson, 1979). It was concluded in similar study that if denaturation and aggregation occur together, an opaque and less elastic gel will result (Schmidt, 1981).

Following equations (5.9 and 5.10) are polynomial models developed for η and Ea of egg white based on CCRD as function of processing parameters (Pressure in MPa; Temperature in °C; Time in minutes). These models can be used to generate η and Ea of WLE under different experimental conditions.

$$\text{Viscosity index} = 442 - 1.75*P - 4.549*t - 8.017*T + 2.271E\text{-}03*P^2 + 0.0804*t^2 + 0.139*T^2 + 5.9234E\text{-}003*P*t + 2.65E\text{-}003*P*T + 0.037*t*T \quad (R^2=0.99) \quad \textbf{(5.9)}$$

$$\text{Apparent elasticity} = 135.47 - 0.46*P - 1.06*t - 2.93*T + 6E\text{-}004*P^2 + 2.09E\text{-}003*t^2 + 0.049*T^2 + 1.68E\text{-}003*P*T + 7.5E\text{-}004*P*T + 0.015*t*T \quad (R^2=0.97) \quad \textbf{(5.10)}$$

5.4.3 Comparison of viscosity index (Back Extrusion) and apparent viscosity (rheology)

The rheological and textural properties of egg components (EW, EY and WLE) are of prime importance to their own quality and as a functional ingredient in different food products. The improvement of quality through new product development requires understanding of factors affecting rheological and textural properties. Knowledge of rheological properties of egg components are of immense significance for efficiency of manufacturing. Similarly, textural properties are used to determine viscosity index and consistency index. In depth assessment of these properties requires their analysis by some established method for their quantification and qualification (Bourne, 2002).

In this part, different methods were employed for evaluation of relationship between apparent viscosity (rheological property) and viscosity index (textural property).

Apparent viscosity value was determined at shear rate of 100 s^{-1}. All processing factors (P, t and T) were found to affect apparent viscosity in significant manner. Effect of increasing pressure was directly proportional to apparent viscosity. Apparent viscosity was influenced significant by all processing factors. Regression analysis conducted on the data divulged high and significant R^2 value for all egg components. Regression model used here showed very high $R^2 > 0.90$, indicating that these two rheological parameters were highly related and could individually be used to predict rheological behavior of egg components.

Figures 5.4, 5.5 and 5.6 show the respective regression models of egg white, egg yolk and whole liquid egg. The observed high positive correlation (>95%) and regression coefficients (≥93%) between apparent viscosity and viscosity index which suggests that viscosity of index could be measured by back extrusion technique using texture analyzers to predict apparent viscosity during manufacturing of different food products (Afoakwa et al., 2008).

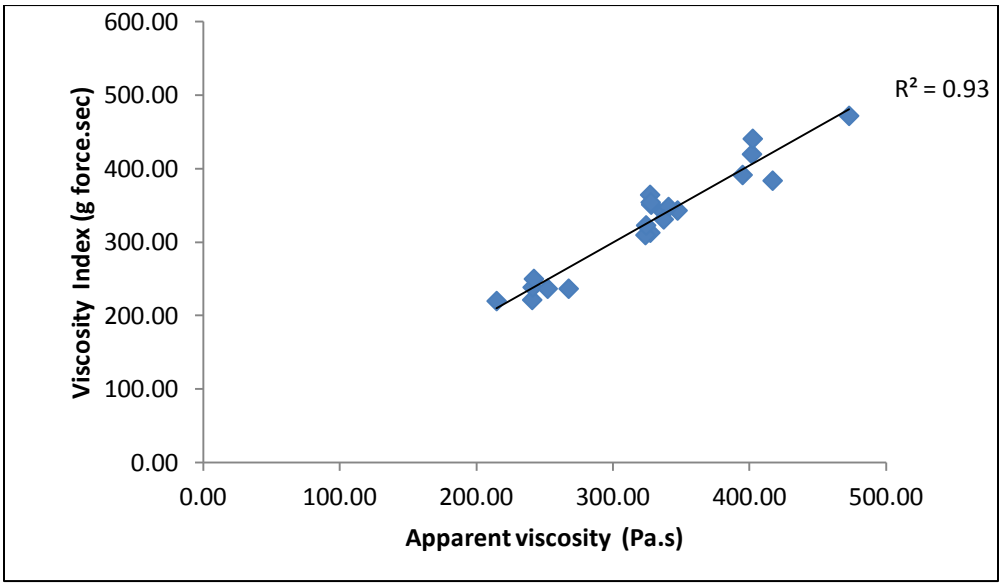


Figure 5.4: Relationship between viscosity index and apparent viscosity of egg white Data points (*squares*); linear regression (*inner solid line*)

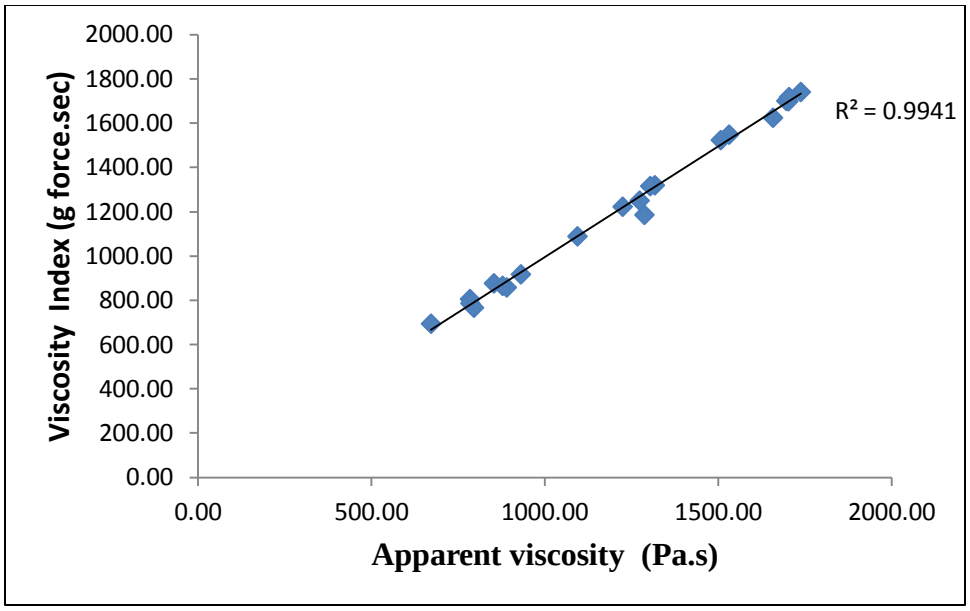


Figure 5.5: Relationship between viscosity index and apparent viscosity of egg yolk. Data points (*squares*); linear regression (*inner solid line*)

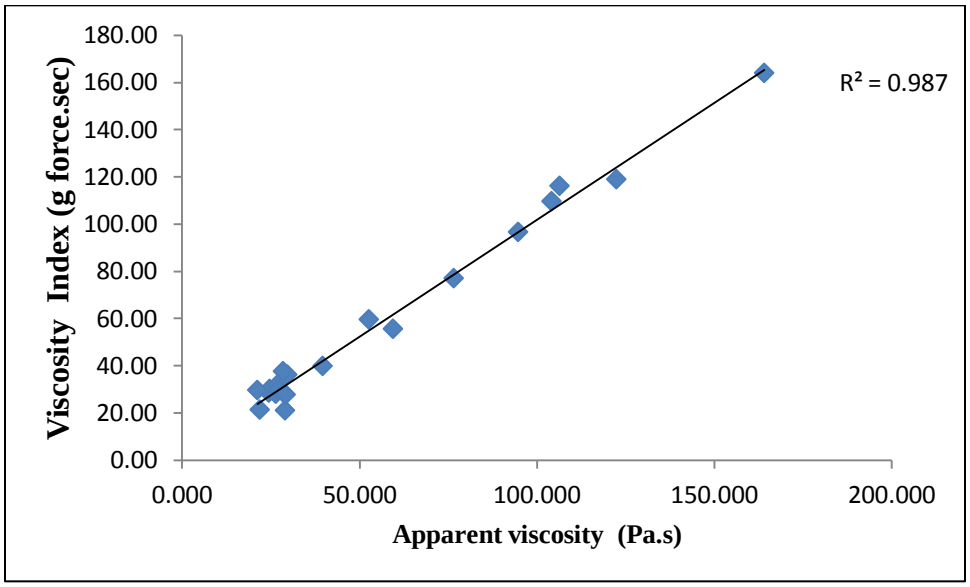


Figure 5.6: Relationship between viscosity index and apparent viscosity of whole liquid egg. Data points (*squares*); linear regression (*inner solid line*)

The relationship between textural properties (viscosity index) and rheological properties (apparent viscosity) showed high highly positive correlation for all egg components (egg white, egg yolk and whole liquid egg) as it was found to be r = 0.96, 0.99 and 0.99. Similarly regression coefficient was found to be R^2= 0.93, 0.99 and 0.98 for EW, EY and WLE. The purpose was to establish the extent to which both rheological and textural properties can be used to correlate the behavior of EW, EY and WLE and could be used to predict the behavior of their respective products during consumption. This knowledge would be useful for new product development and process engineering purposes.

Though there are number of factors which influence the characteristic of egg, these results suggested that these particular factors have high accountability. It was found that these characteristics (apparent viscosity and viscosity index) were dependent on composition of egg components.

Egg yolk is a complex association of lipids (33% by weight), proteins (17%) and water (50%) suspended in protein solution (Shenstone, 1968). Thus egg yolk being high in fat showed increase in viscosity with increase in treatment intensity where as EW shows protein coagulation with increase in treatment intensity and was found to show inconsistent behavior as it was high in protein. The possible reason for EW behavior could be dependent on denaturation and coagulation which in turn is dependent on type of protein, pressure and temperature used (Ahmed et al., 2003). On other hand, WLE being a combination of EW and EY showed altogether different behavior. These results were in close agreement with Afoakwa et al., (2007) which reported that apparent viscosity was more dependent on fat and lecithin contents in case of chocolate.

In modern day egg and egg product manufacturing, the need for tightly controlled processes aiming at utmost effectiveness and utilization levels necessitates understanding factors influencing textural and rheological properties of egg components as well as their derived food products. Such information would certainly enhance quality assurance and process design.

5.5 Conclusions

The effect of high pressure in combination with temperature and time treatment on back extrusion properties of egg components was studied. It was found that mostly all processing factor affect back extrusion of egg components in a significant manner. High pressure caused coagulation of egg white. Protein denaturation was dependent on intensity of treatments (pressure level, treatment time and temperature) used as observed from differences in product under observation. Egg yolk showed increased in viscosity with increase in intensity of pressure. Correlation between viscosity index and apparent viscosity showed that rheological and back extrusion properties can be used in comparative manner. Further research is required for correlation between pressure induced back extrusion and functional properties of egg components.

PREFACE

TO CHAPTER 6

In earlier studies, we have studied the rheological properties of all liquid and semi solid phase for egg components. Pressure treatment of egg components at different conditions can cause partial unfolding of proteins. This protein unfolding can lead to the reversible or irreversible gelation of the product, with repercussions on the color and textural characteristics of the egg components. There is great industrial interest; as even just a modulation of the rheological parameters of the egg components with the pressure can cause formation of desirable hard gels due to extensive modification of the protein's secondary and tertiary structures.

One of the primary purposes with this egg component employed in this study was to explore the possibility of using them as functionality enhancing ingredients with the use of high pressure to facilitate a structure-altering process with potential applications in other food applications.

Part of this research has been presented in the annual meeting of the Institute of food technologists in Chicago, USA in July 2010.

Singh A and Ramaswamy HS (2012) Effect of high pressure processing on physico-chemical characteristics of egg components (Prepared for Submission).

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy

CHAPTER 6

EFFECT OF HIGH PRESSURE PROCESSING ON PHYSICO-CHEMICAL CHARACTERISTICS OF EGG COMPONENTS

6.1 Abstract

This work deals with effect of High pressure processing (HPP) on physicochemical characteristics like color and texture of various egg components like egg white (EW), egg yolk (EY) and whole liquid egg (WLE). HPP can cause denaturation of egg white and whole liquid egg leading to coagulation and gelation with increase in pressure level. A full factorial design involving various pressure levels (600-900MPa) and treatment time (0-15 min) was employed for this study. Color and textural changes were studied using Minolta colorimeter and TA.TX2 texturemeter respectively. High pressure was found to cause significant changes in various egg components and pressure level of 600 MPa or more was able to generate fully formed gels. Pressure induced gels were soft, highly elastic and without any cooked flavor and taste. Total color difference (ΔE) showed a significant ($p < 0.05$) increase in its value with increase in pressure level and treatment time. Textural properties (hardness and cohesiveness) were found to be increasing significantly ($p < 0.05$) with increase in treatment intensity for all egg components, but increase in EY was higher than other egg components.WLE followed a different trend for springiness as by increasing pressure and treatment time, it had higher value than EW and EY. Other textural and color parameters showed similar observations for various egg components.

6.2 Introduction

Consumer trends in today's food market are preferably inclined more towards minimally processed, fresh like and additive free foods. Due to these trends, many novel food processing technologies have been investigated as a supplementary process or as alternative methods to conventional food processing techniques. These methods include ohmic heating, radio frequency, microwave heating, ultrasound, high voltage arc

discharge or high pressure processing (HPP). In all these methods, HPP has demonstrated repeatability to act as an alternative method to satisfy microbiological stability and to impart fresh like characteristics to foods as it was found that HPP can affect only non covalent bonds, causing unfolding of protein chains and little effect on chemical constituents associated with desirable food qualities such as flavour, color and nutritional content (Hayashi, 1990); whereas heat treatments can cause change in covalent bonds which can increase the production of off - flavors or toxic compounds (Ngarize et al.,2005). HP causes rupture of non-covalent interactions within protein molecules followed by subsequent re formation of intra molecular and intermolecular bonds within protein molecules.

High pressure processing (HPP) is a prospective non thermal alternative for pasteurization of low acid food products. The HPP treatment can result in product stabilization and microbial destruction without affecting their sensory qualities (Basak and Ramaswamy, 1998). It was found that high pressure can change protein conformation of egg which can cause denaturation, aggregation or gelation, depending on the protein system, the applied pressure and duration of the pressure treatment (Messens et al., 1997). This approach can play a vital role in developing egg patties without the use of heat as coagulation and aggregation follow each other at high pressure level. Thus egg patty with natural and fresh characteristics without any cooked flavor can be made using HPP, Egg is a versatile food ingredient used in number of food preparations. Bridgeman, (1914) found that egg white gets fully coagulated to form gels, when subjected to 600 MPa pressure. Egg can supply all essential amino acids for humans, and provide several vitamins and minerals, including vitamin A, riboflavin, folic acid, vitamin B_6, vitamin B_{12}, choline, iron, calcium, phosphorus and potassium. The egg is a low acid food (higher pH) which necessitates preservation by some means to increase the shelf life. Conventional thermal methods are used for its preservation, but they are not very highly efficient as it can cause reduction in nutritional and sensory quality of egg by giving cooked flavor (Hayashi et al., 1989).

This technology offers supplementary advantage of preserving sensory quality after processing of egg and egg products as egg proteins have delicate sensory properties,

which are often adversely influenced by conventional heat treatments used for their pasteurization. High pressure doesn't affect the covalent bonds responsible for flavour and other volatiles but it can cause rupture of non covalent interactions within protein molecules which leads to formation of intermolecular bonds between protein molecules. These different types of intermolecular bonds cause the stabilization of secondary, tertiary and quaternary structure of proteins (Messens et al., 1999). Texture is considered one of the most important sensory attributes in determining the overall quality, which often undergoes considerable alteration during the thermal pasteurization. HPP is known to cause elimination of vegetative micro organisms in diminutive time than conventional thermal process (Juliano et al., 2006).

It was found that HP level ranging from 400 - 600 MPa can cause the resulting material to become gel with improved quality characteristics than the heat induced gels. Pressure induced gels were softer and more elastic without any cooked taste and flavour and there was no destruction of vitamins and formation of lysinoalanine. Based on these results, it can be anticipated that high pressure is useful in food processing and can cause preservation to circumvent unfavourable effects on natural foods (Hayashi et al., 1989). Ovalbumin represents major protein in egg white, and it plays a major role in determining egg white behavior to high pressure. HPP modification results in structural changes in egg white which can be correlated to functional properties. This modification influences the SH groups present in ovalbumin. Thiol group plays a critical function in the formation of protein aggregates and in the stabilization of a gel structure upon heat treatment, which most likely transpires through disulfide exchange mechanism (Mine, 1997).

There are number of studies that have been published with the scope indicating potential applications and limitations of high-pressure technology for egg gels (Okomato et al., 1990; Hayashi, 1990; Balny and Masson, 1993; Van Camp and Huyghebaert, 1995; Messens et al., 1997) but it still lacks proper evaluation of HPP effect on physico-chemical characteristics of egg. These characteristics play an imperative role in behavior of egg proteins during their processing, storage and maintaining sensory attributes. Hence, exploration of physico chemical characteristics of these egg components will

assist in revealing structure and function relationship for further use of egg as whole and as an ingredient in other products.

The objective of this study was to evaluate effect of high pressure processing on physico-chemical properties of egg components as it changes from highly viscous phase to hard gels (egg patty) with increase in intensity of the high pressure level used. In order to evaluate HP process in terms of physical changes in egg patty, texture profile analysis was done so as to simulate actual texture behavior of egg components in mouth and color analysis was done to assess changes in color of egg components.

6.3 Materials and methods

6.3.1 Sample Preparation

Large sized fresh eggs were bought from local grocery store. They were washed and sorted to remove any dirty or leaker eggs. Egg white was separated from egg yolk by breaking the egg on the tip and allowing the egg white to flow out leaving the intact egg yolk inside. The intact egg yolk (EY) was poured over tissue paper to remove any residue of egg white (EW), if present. The egg was broken and mixed properly for its use as a whole liquid egg (WLE). These samples were poured into low density 2 oz. polyethylene bags (Whirl Pak$^{(R)}$, USA) and sealed using heat sealer. The samples were then stored at 4°C, removed and equilibrated to the test conditions (20°C) just prior to pressure treatment as detailed in the next section.

6.3.2 High pressure processing treatments

Samples were then transferred to a 5L cylindrical pressure treatment chamber (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France). Pure water was used as pressurizing medium in the HP unit. Egg samples were subjected to pressure treatments as shown in Table 6.1. Pressurization and depressurization rate of 4.4 MPa/s and 26 MPa/s respectively, were used during high pressure treatments. Pressure come up and pressure release times were not considered in actual processing time. High pressure level (>500 MPa) were used to form egg patty with better functional properties as it doesn't involve application of any extenal heat. All the experiments were carried out in duplicate.

Pressure treated samples were immediately transferred to refrigerator (4°C) until texture and color measurements were made.

6.3.3 Color measurement

The surface color of treated egg samples was evaluated in L, a, and b units using a tristimulus Minolta CM-508d spectrophotometer (Minolta Co, Japan). The instrument was warmed up for 10 min before the actual measurements and calibration was performed using a white standard plate. Five measurements were made individually for each sample, and the average value was reported. The color value was determined in a three-dimensional color space, L (luminosity), a (green − to red +), and b (blue− to yellow +) values of the egg samples. In addition, the total color difference, ΔE (1), and Hue angle (3) and Chroma were (2) computed from the L, a, and b values. Raw egg components were used as the reference for measuring ΔE, where the subscript "o" refers to the color reading of raw egg component:

$$\Delta E = \sqrt{(L_0\text{*-L*})^2 + (a_0\text{*-a*})^2 + (b_0\text{*-b*})^2} \quad (6.1)$$

$$Chroma = \sqrt{(a*^2 + b*^2)} \quad (6.2)$$

$$Hue\ angle = tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (6.3)$$

6.3.4 Texture profile analysis

TA.XT plus texture meter with 2 kg load cell (Texture technologies corp, Scarsdale, NY, USA) was used to evaluate texture profile analysis (TPA) (Figure 6.1) for the following measurements:

1. Hardness (maximum force required to compress the sample)
2. Adhesiveness (work necessary to pull the compression anvil away from the sample),

3. Springiness (the height that the food recovers during the time that elapses between the end of the first bite and the start of the second bite), and
4. Cohesiveness (the ratio of the positive force area during the second compression portion to that during the first compression (Area 2/Area l)

The probe employed in this study was TA-25 probe (2"diameter cylinder, aluminum, and 20 mm tall). All experiments were carried out at room temperature (20°C). In this study, samples were compressed up to 25% of their original height to calculate TPA. Pre-test, test and post test speed of 1.0, 1.5 and 1.5 m/s respectively were used for this test. The instrument automatically recorded the force–displacement or force–time curve and converted them to texture profile analysis. Five to six replicates were conducted for each set of experiment. The compression device was larger than the sample size for TPA, so it confers that forces registered in TPA are largely due to uniaxial compression forces (Bourne, 2002)

6.3.5 Statistical analysis

A full scale factorial design was used employing 4 levels of pressure (600-900 MPa) and time (0-15min) as explained in Table 6.1. ANOVA analysis was performed on the data using two way ANOVA with help of Minitab 16 Statistical Software (Minitab Inc, State College, Pa, USA). ANOVA analysis was done and significance level was reported with 95% level of confidence. It was performed to evaluate effect of treatment factors (time and pressure) and their combination on the color and textural parameters.

Table 6.1: Full factorial design combining pressure level and treatment time used

Pressure Level (MPa)	Treatment Time (minutes)			
600	0	5	10	15
700	0	5	10	15
800	0	5	10	15
900	0	5	10	15

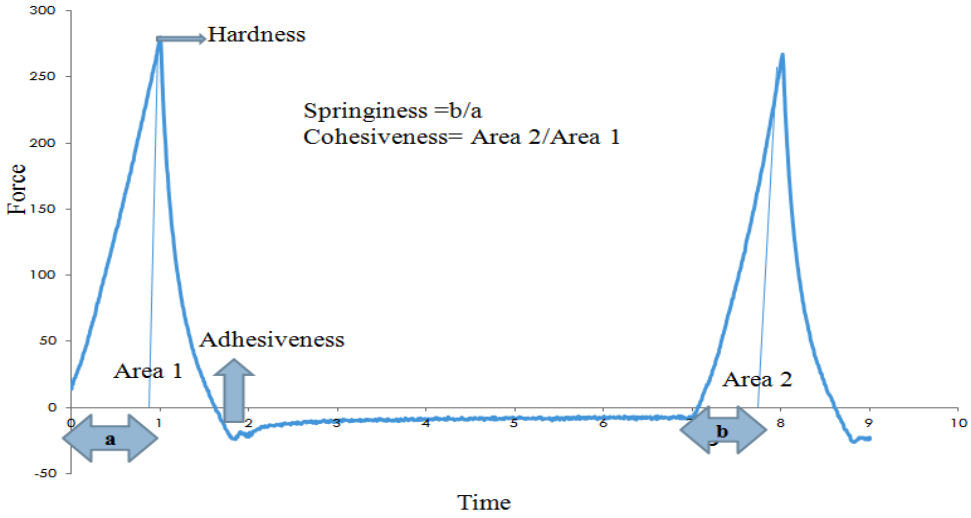


Figure 6.1: Typical texture profile analysis showing measurement of various parameters

6.4 Results and discussions

6.4.1 Effect of HP treatment variables on color parameters of egg components

6.4.1.1 Color (L, a and b)

High pressure processing caused number of changes in the color values of egg white, egg yolk and whole liquid egg respectively (Table 6.2). For all egg components, there were significant (p <0.05) changes caused in their color parameters with variation of the process variables.

For egg white, it was found that L* value increased linearly at all pressure-time combinations indicating increase in brightness of sample with increasing pressure treatment intensity. ANOVA analysis showed that both treatment factors (pressure level and treatment time) and their interaction effect were highly significant (p <0.05) in terms of affecting L* value. In similar way, a* value followed the identical trend as L* while the increase in a* value (indicating the redness of the sample) was minuscule in comparison to L* value. ANOVA analysis suggested that treatment factors and their

interaction affected a* value in a significant way. Similarly, there was small increase in b* value from 1.91±0.00 to 2.214±0.002, though it was indicated by ANOVA analysis that this change was significant (p <0.05) (Table 6.3). ANOVA analysis shows that in all egg components, R^2 was found to be > 0.95 indicating high significance of the two way ANOVA model used (Table 6.3).

Table 6.2: Hunter color values of the egg white subjected to pressure level and treatment time

Egg White	Time→	0	5	10	15
	Press. Level↓				
L* value	**600**	58±0.707	63.5±3.5	66±4.24	80±1.14
	700	81±1.41	86	85.5±0.70	91.5±0.70
	800	86±3.53	89±4.24	91.5±2.12	96±1.41
	900	88±2.82	91.5±3.5	94±1.41	100.5±0.70
a* value	**600**	1.2±0.007	2.005±0.021	2.22±0.04	2.26±0.014
	700	2.8±0.077	2.91±0.01	3.125±0.035	3.13±0.01
	800	1.94±0.65	2.065±0.021	2.005±0.007	2.35±0.070
	900	1.9±0.02	2.095±0.021	20.025±0.007	2.25±0.07
b* value	**600**	1.9	1.61±0.014	1.805±0.0007	2.25±0.07
	700	1.5±0.14	1.19±0.014	1.11±0.014	1.25±0.07
	800	1.3±0.07	1.11±0.014	0.98±0.0141	0.855±0.021
	900	0.99±0.01	1.085±0.021	1.615±0.021	2.214±0.0021

Table 6.3: ANOVA analysis indicating significance of treatment factors on color of EW

Two-way ANOVA: L* value versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	682	227.33	37.89	0.000
Pressure	3	3655	1218.33	203.06	0.000
Interaction	9	159	17.67	2.94	0.029
Error	16	96	6.00		
Total	31	4592			

S = 2.449 R-Sq = 97.91% R-Sq (adj) = 95.95%

Two-way ANOVA: a* value versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	1.81666	0.60555	21.78	0.000
Pressure	3	6.26798	2.08933	75.15	0.000
Interaction	9	0.68818	0.07646	2.75	0.037
Error	16	0.44485	0.02780		
Total	31	9.21767			

S = 0.1667 R-Squared = 95.17% R-Squared (adjusted) = 90.65%

Two-way ANOVA: b* value versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	0.65208	0.217361	91.40	0.000
Pressure	3	2.89501	0.965003	405.78	0.000
Interaction	9	2.25588	0.250653	105.40	0.000
Error	16	0.03805	0.002378		
Total	31	5.84102			

S = 0.04877 R-Sq = 99.35% R-Sq (adj) = 98.74%

Egg Yolk being high in xanthophylls (yellow color) showed altogether different color behavior than that of egg white. Table 6.4 presents the behavior of L*, a* and b* values in response to pressure treatments. Egg yolk showed increase in viscosity and its color changed from pale yellow to orangish yellow as per visual appearance. L* value remained constant showing a minute increase from 57.4 to 58.5 only where as a* value was found to be decreasing from 9.51 to 2.11 indicating diminution in redness of sample but on the other hand, b* value increased significantly (p <0.05) from 56.5 to 76.5 showing great deal of increase in yellow color of the egg yolk making it more appealing from a customer viewpoint. High pressure level of 900 MPa showed the maximum L*

and b* values of treated samples. In similar fashion to EW, it was found that R^2 values were greater than 0.95% indicating higher efficacy of ANOVA (2 way model) used (Table 6.5).

Table 6.4: Hunter color values of the egg yolk treated with pressure level and treatment time

Egg Yolk	Time→	0	5	10	15
	Press. Level↓				
L* value	**600**	57.4±0.84	56.8±0.28	53.85±0.21	61.1±0.14
	700	57.05±1.34	55.7±0.49	51.75±0.35	44.8±0.070
	800	55.3±0.98	50.6±0.84	50.6±0.56	51.25±0.07
	900	57±1.41	53.15±0.21	55.1±0.28	58.5±0.28
a* value	**600**	9.51±0.014	8.46±0.05	8.405±0.007	61.5±0.07
	700	8.63±0.04	5.84±0.06	5.36±0.05	4.15±0.07
	800	4.15±0.07	2.95±03.07	2.6±0.28	1.85±0.07
	900	3.95±0.07	2.765±0.091	2.36±0.06	2.11±0.014
b* value	**600**	44.19±15.2	56.15±1.20	58.1±0.14	58.1±0.14
	700	61.1±0.14	62.5±0.70	62.25±1.06	63.4±1.27
	800	64.75±0.35	66.5±3.53	70.4±0.28	70.6±0.28
	900	69.3±1.83	70.5±2.12	72.5±2.12	75.5±0.70

L *, a* and b* values of whole liquid egg are shown in Table 6.6. L* value of WLE amplified significantly (p <0.05) from 47.9 to 68.3 showing a great deal of increase in lightness of sample and it was well observed through visual observation. In similar way, a* and b* value were found to be rising significantly (p <0.05) from 15.45 to 27.1 and 31.55 to 45.4 respectively which indicated that whole egg samples were more attractive with increased lightness and reddish yellow color.

Effect of pressure level, treatment time and their interaction was found to be highly significant (Table 6.7). The main possible reason for changes in egg samples could be rearrangement of water molecules after pressure treatment that tend to modify the gel network, exhibiting a more lustrous and transparent appearance than heat-induced gels. (Munizaga and Barbosa, 2004)

Table 6.5: ANOVA results indicating effect of treatment factors on color of egg yolk

Two way Anova L* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	64.648	21.5495	47.39	0.000
Pressure	3	169.366	56.4553	124.16	0.000
Interaction	9	233.783	25.9759	57.13	0.000
Error	16	7.275	0.4547		
Total	31	475.07			
S = 0.6743 R-Squared = 98.47% R-Squared (adj) = 97.03%					
Two way Anova a* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	36.680	12.2267	1455.01	0.000
Pressure	3	160.880	53.6268	6381.77	0.00
Interaction	9	6.383	0.7092	84.40	0.000
Error	16	0.134	0.0084		
Total	31	204.078			
S = 0.09167 R-Squared = 99.93% R-Squared (adj) = 99.87%					
Two way Anova b* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	114.69	38.228	21.42	0.000
Pressure	3	916.81	305.602	171.21	0.000
Interaction	9	52.82	5.869	3.29	0.018
Error	16	28.561.785			
Total	31	1112.88			
S = 1.336 R-Squared = 97.43% R-Squared (adj) = 95.03%					

Table 6.6: Impact of treatment factors on color value of whole liquid egg

Whole liquid egg	**Time→**	**0**	**5**	**10**	**15**
	Press. Level↓				
L* value	**600**	47.9±0.014	48.65±0.49	53.4±0.56	58.6±0.84
	700	53.6±0.56	53.55±0.77	55.1±0.28	61.8±0.14
	800	57.15±0.212	61.35±0.49	65.5±0.70	65.55±0.21
	900	59.15±0.212	65.15±0.21	67.9±0.14	68.3±0.14
a* value	**600**	15.45±0.07	16.15±0.21	19.1±0.14	25.15±0.21
	700	17.15±0.212	18.1±0.14	19.7±0.28	23.5±0.70
	800	19.05±0.212	18.15±0.21	21.2±0.28	24.8±0.70
	900	19.7±0.42	20.6±0.14	21.55±0.21	27.1±0.28
b* value	**600**	31.55±0.07	32.05±0.07	34±0.28	37.5±0.70
	700	34.35±0.21	35.1±0.42	36.15±0.21	37±0.14
	800	35.1±0.141	37.15±0.21	39.65±0.63	41.35±0.07
	900	38.15±0.07	39.6±0.141	40.7±0.14	45.4±0.28

Table 6.7: ANOVA indicating significance of treatment factors on color of WLE

Two way Anova L* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	375.97	125.324	608.56	0.000
Pressure	3	839.85	279.950	1359.39	0.000
Interaction	9	66.76	7.418	36.02	0.000
Error	16	3.30	0.206		
Total	31	1285.88			
S = 0.4538 R-Sq = 99.74% R-Sq (adj) = 99.50%					

Two way Anova a* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	269.063	89.6878	817.67	0.000
Pressure	3	49.783	16.5945	151.29	0.000
Interaction	9	13.948	1.5498	14.13	0.000
Error	16	1.755	0.1097		
Total	31	334.550			
S = 0.3312 R-Squared = 99.48% R-Squared (adj) = 98.98%					

Two way Anova b* value v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	137.493	45.8308	495.47	0.000
Pressure	3	236.197	78.7325	851.16	0.000
Interaction	9	18.770	2.0856	22.55	0.000
Error	16	1.480	0.0925		
Total	31	393.940			
S = 0.3041 R-Sq = 99.62% R-Sq (adj) = 99.27%					

In other similar studies, it was found that egg gels treated with high pressure keep their original taste without any cooked flavor (Okomato et al., 1989). Even in case of other protein rich foods, it was found that the HPP can cause many changes in fish muscle in terms of color and texture (Zhu and Ramaswamy, 2004). There are mainly 3 carotenoids which are responsible for yellow color in the egg products: Lutein, zeaxanthin and cryptoxanthin (Li-Chan et al., 1995) and HPP modify these pigments in such a way that modifies the color characteristics of egg gels.

6.4.1.2 Color (ΔE, Hue angle and Chroma)

ΔE is the total color difference which was obtained as the combined differences in L*, a*, and b* values (Eq.6.1) and has been comprehensively employed to present the color variance of foods during processing (Azarpazhooh and Ramaswamy, 2011). ΔE

was calculated using L*, a* and b* values whereas raw egg components acted as reference. ΔE was quite significantly (p<0.05) affected by all process variables and their interaction. Hue angle (H) was measured as anue of red, yellow and blue color. A hue angle > 90° indicates yellowish green color whereas Hue angle < 90° indicates more orange color. Chroma depicts the strength of color and it is related to degree of saturation. Ahmed et al. (2005) found that ΔE remained constant even after increase in HP treatment indicating stability of pigments.

In EW, ΔE value increased linearly from 600-900 MPa with increase in treatment time rising from 0-15 min (Table 6.8). ΔE value showed an approximate 100% increase as it elevated from 57.5 to 103.8 accompanied by visual change in color of egg white. The EW coagulated with increase in pressure level and treatment time, thereby changing its color from slightly transparent to opaque. The main factor responsible for change in color of egg white could be impact of high pressure on texture as structure and pigments of food interact with each other to affect color and translucency/opacity (Oey et al., 2008).

Table 6.8: Effect of HP treatments on ΔE, Hue angle and Chroma values of EW

Delta E (ΔE) **Time→**	**0**	**5**	**10**	**15**
Press. Level↓				
600	57.51±0.707	63.50±3.34	68.12±3.86	83.43±1.72
700	89.65±2.37	93.92±0.13	98.60±1.08	101.72±0.6
800	84.37± 1.10	89.08±4.42	91.05±2.17	98.54±0.77
900	88.58±2.65	91.83±3.70	94.17±1.51	103.76±0.03
Hue angle	**0**	**5**	**10**	**15**
600	57.801±0.41	38.764±0.05	39.116±0.42	44.866±0.72
700	29.321±1.49	22.241±0.33	19.554±0.02	21.767±1.2
800	43.875±1.42	28.259±0.05	26.048±0.40	20.004±1.01
900	27.469±0.074	27.38±0.69	38.572±0.27	44.557±0.62
Chroma	**0**	**5**	**10**	**15**
600	2.24±0.03	2.57±0.025	2.86±0.03	3.18±0.05
700	3.26±0.13	3.14±0.07	3.31±0.03	3.37±0.01
800	2.03±0.42	2.34±0.025	2.23±0.001	2.5±0.06
900	2.12±0.02	2.35±0.009	2.59±0.01	3.15±0.06

In EW, Hue angle showed opposite trend to that of ΔE. It was found using ANOVA analysis that treatment factors and their interaction was highly significant (p<0.05) in affecting Hue angle. Increase in pressure level and treatment time range caused decrease in Hue angle but decrease was very minimal in comparison to increase in ΔE values. There was small yet significant (p<0.05) increase in Chroma value with increase in processing treatment level (Table 6.9). High pressure affects the textural properties which in turn can affect the nature and extent of scattered light and distribution of surface reflectance leading to color change (MacDougall, 2002).

Table 6.9: ANOVA indicating significance of treatment factors on color of EW

Two way Anova ΔE v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	1390.94	463.65	86.37	0.000
Pressure	3	4494.18	1498.06	279.07	0.000
Interaction	9	259.13	28.79	5.36	0.002
Error	16	85.89	5.37		
Total	31	6230.13			
S = 2.317 R-Sq = 98.62% R-Sq (adj) = 97.33%					
Two way Anova Chroma v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	1.73195	0.57732	42.47	0.000
Pressure	3	4.22425	1.40808	103.59	0.000
Interaction	9	0.69782	0.07754	5.70	0.001
Error	16	0.21749	0.01359		
Total	31	6.87151			
S = 0.1166 R-Sq = 96.83% R-Sq (adj) = 93.87%					
Two way Anova Hue angle v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	498.46	166.154	12.74	0.000
Pressure	3	2042.34	680.782	52.21	0.000
Interaction	9	1125.15	125.017	9.59	0.000
Error	16	208.61	13.038		
Total	31	3874.57			
S = 3.611 R-Sq = 94.62% R-Sq (adj) = 89.57%					

In EY, ΔE was found to be increasing with increase in pressure level and treatment time (Table 6.10). ANOVA analysis states that both treatment factors and their interaction had significant (p<0.05) affect on ΔE value. There was nominal but significant (p<0.05) increase in Hue angle from 80.21±0.22 to 88.4±0.01 with increase in treatment intensity but on the other hand, Chroma values increased significantly from 55.96±0.21 to 76.03±0.03 which was found to be highly significant (p<0.05) (Table 6.11)

Table 6.10: Effect of HP treatments on ΔE, hue angle and chroma values of EY

Delta E (ΔE) **Time→**	**0**	**5**	**10**	**15**
Press. Level ↓				
600	520±10.17	621.9±34.1	672.8±28.8	808.1±8
700	831.8±41.3	911.8±0.43	850.6±40.9	990.4±9
800	1007.4±43.3	1352± 62.8	1435.9±9	1427.90±11.2
900	1274.9±50.7	1497.8±53	1740.5±57	1871.3±0.16
Hue angle	0	5	10	15
600	80.21±0.22	81.62±0.14	81.83±0.07	84.25±0.07
700	82.01±0.05	84.69±0.05	85.05±0.10	86.31±0.07
800	86.31±0.10	87.57±0.08	87.89±0.22	88.49±0.06
900	86.69±0.024	87.78±0.05	88.18±0.03	88.4±0.01
Chroma	0	5	10	15
600	55.96±0.21	58.11±0.69	59.19±0.56	61.40±0.13
700	62.10±0.70	63.27±0.05	62.23±0.69	64.53±0.13
800	64.63±0.70	69.66±0.84	70.74±0.15	70.52±0.13
900	68.61±0.71	71.55±0.71	74.53±0.70	76.03±0.03

Table 6.12 presents the variation of color characteristics in WLE that showed a different pattern from EW and EY due to difference in their composition and their response to HPP. ΔE value increased significantly (p <0.05) from 190.4±2.38 to 921.77±3.90. There was tremendous increase in ΔE (approximately 5 fold) which is much higher than that of EW and EY. Small decrease in Hue angle (63.9±0.052 to 59.16±0.42) was observed indicating decrease in yellowish color of the product but ANOVA analysis indicated that even this smaller decrease was highly significant (p <0.05). On the other hand, Chroma

increased from 35.13±0.09 to 52.87±0.09 which was highly significant (p <0.05), but it was lower than EY and higher than that of EW (Table 6.13).

Table 6.11: ANOVA analysis indicating significance on color of egg yolk

Two way Anova ΔE v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	36.426	12.1419	651.05	0.000
Pressure	3	178.708	59.56923	194.11	0.000
Interaction	9	6.411	0.7123	38.19	0.000
Error	16	0.298	0.0186		
Total	31	221.842			
S = 0.1366 R-Sq = 99.87% R-Sq (adj) = 99.74%					
Two way Anova Chroma v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	102.92	34.306	19.37	0.000
Pressure	3	847.53	282.511	159.49	0.000
Interaction	9	55.71	6.190	3.49	0.014
Error	16	28.34	1.771		
Total	31	1034.50			
S = 1.331 R-Sq = 97.26% R-Sq (adj) = 94.69%					
Two way Anova Hue angle v/s Time and Pressure					
Source	DF	SS	MS	F	P
Time	3	36.426	12.1419	651.05	0.000
Pressure	3	178.708	59.5692	3194.11	0.000
Interaction	9	6.411	0.7123	38.19	0.000
Error	16	0.298	0.0186		
Total	31	221.842			
S = 0.1366 R-Sq = 99.87% R-Sq(adj) = 99.74%					

From these results, it was observed that HPP can cause changes in color of egg components which can be related to changes in textural properties as they changed from viscous liquid to fully formed gels on increasing intensity of pressure treatment. This similar phenomenon was observed in another study on tomato products, where HP treatment (400 MPa/25 °C/15 min) caused increase in L* value of surface color of puree indicating increase in lightening of puree surface. The possible reason could be the formation of jelly like viscous translucent tomato puree when high pressure processing

was used (Verlent et al., 2006). It was found that changes in color appearance would be more expected rather than the changes in pigment concentration (Mac Dougall, 2002).

Table 6.12: Effect of process variables on ΔE, hue angle and chroma values of WLE

Delta E (ΔE) Time→	0	5	10	15
Press. Level↓				
600	190.4±2.38	212.79±5.6	323.12±1.93	613.82±23.31
700	267.87±8.06	305.56±0.67	373.79±13.14	537.21±31.51
800	338.97±9	346.9±10.73	504.32±25.52	690.89±28.97
900	413.26±15.4	480.96±8.42	544.4±11.57	921.77±3.90
Hue angle	0	5	10	15
600	63.9±0.052	63.25±0.25	60.67±0.38	56.15±0.27
700	63.47±0.14	62.71±0.46	61.41±0.20	57.58±0.68
800	61.51±0.17	63.96±0.13	61.86±0.06	59.04±0.76
900	62.69±0.46	62.51±0.07	62.09±0.15	59.16±0.42
Chroma	0	5	10	15
600	35.13±0.094	35.88±0.15	38.99±0.17	45.15±0.70
700	38.39±0.28	39.49±0.31	41.16±0.32	43.83±0.49
800	39.94±0.22	41.34±0.28	44.96±0.69	48.21±0.30
900	42.94±0.25	44.63±0.19	46.05±0.22	52.87±0.09

Table 6.13: ANOVA analysis indicating significance of ΔE on whole liquid egg

Two way Anova ΔE v/s Time and Pressure

Source	DF	SS	MS	F	P
Time	3	740309	246770	996.13	0.000
Pressure	3	313596	104532	421.96	0.000
Interaction	9	47395	5266	21.26	0.000
Error	16	3964	248		
Total	31	1105264			

S = 15.74 R-Sq = 99.64% R-Sq (adj) = 99.31%

Two way Anova Chroma v/s Time and Pressure

Source	DF	SS	MS	F	P
Time	3	331.971	110.657	902.54	0.000
Pressure	3	281.241	93.747	764.62	0.000
Interaction	9	23.575	2.619	21.37	0.000
Error	16	1.962	0.123		
Total	31	638.750			

S = 0.3502 R-Sq = 99.69% R-Sq (adj) = 99.40%

Two way Anova Hue Angle v/s Time and Pressure

Source	DF	SS	MS	F	P
Time	3	134.657	44.8855	344.66	0.000
Pressure	3	2.059	0.6864	5.27	0.010
Interaction	9	21.565	2.3961	18.40	0.000
Error	16	2.084	0.1302		
Total	31	160.364			

S = 0.3609 R-Sq = 98.70% R-Sq (adj) = 97.48%

6.4.2 Effect of HP treatment variables on texture of egg components

Texture is an imperative characteristic of egg and egg products which affects consumer perception and acceptability. Textural changes in egg components are very sensitive to processing method and parameters used. HPP is a novel food processing method which does not affect the sensory properties of egg components due to limited affect on covalent bonds of low molecular mass compounds. Egg is a rich source of proteins and HPP can be used to modify functionality of egg proteins. The volume of protein decreases due to compression of internal cavities on using high pressure processing but hydration of proteins is reduced which works against this decline in volume thus leading to only small decrease in some proteins (Messens et al., 1999). Thus HPP can be used to modify food proteins in controlled manner so as to make egg gels with better quality, uncooked flavor and better textural properties (Pons and Fiszman, 1996).

Texture profile analysis was performed on pressure treated egg white, egg yolk and whole liquid egg samples. EW showed different behavior than those of EY and WLE due to their high protein content. Application of HPP caused coagulation of egg white and increasing the intensity of pressure level and treatment time caused the gelation of egg white like that of an egg patty. Texture profile analysis of treated samples was done to evaluate Hardness, Adhesiveness, Cohesiveness, Chewiness, Gumminess and Springiness. Egg white turned opaque at 600 MPa and at treatment of 600MPa/15 min was able to form egg gels which can stand by themselves. Egg yolk was able to form gels at 700MPa/15 min and WLE was able to form gels at very short time processing treatment of 700MPa/10 min.

6.4.2.1 Hardness

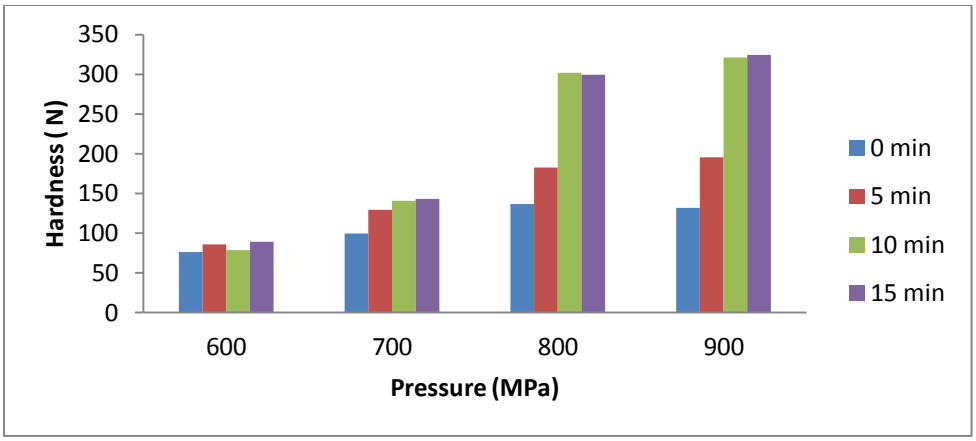
Hardness is mainly known as force required for attaining a given deformation or maximum force which occurs at any time during first compression cycle (Szczesniak, 1963). Hardness was measured to evaluate increasing gelation of egg components. This property was used to depict changes caused in hardness level due to increasing gelation in

various egg components. Egg white proteins called ovalbumin, ovotransferrin, and lyzozyme are responsible for gelation (Johnson and Zabik, 1981).

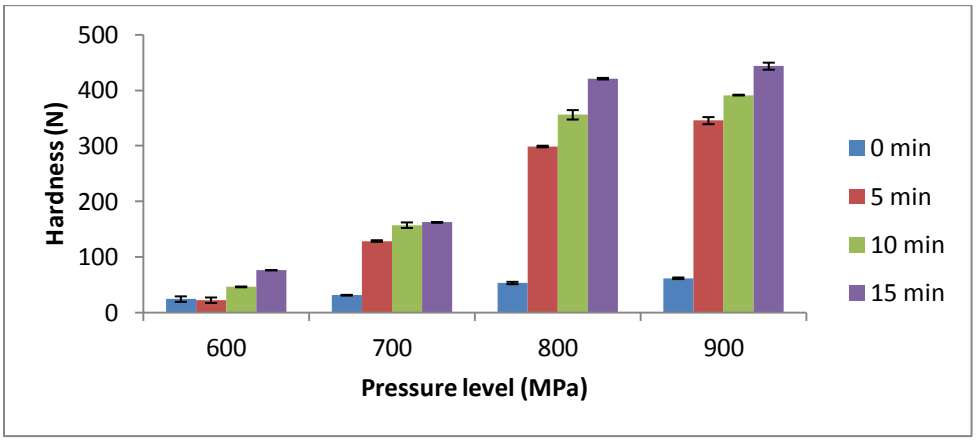
ANOVA was used to evaluate significance of the results. *p* value was used for representing significance level. Smaller *p* value depicts higher significance level. Egg white was found to be showing increasing hardness with increasing pressure and time treatment intensity (Figure 6.2a). Increase in hardness value at higher processing pressure and time were much more higher than that of lower processing treatments which indicates that higher pressure and treatment time are required for proper gelation of egg white into solid patty. Tunick et al. (1991) found that higher level of hardness could be due to reduced moisture level. They hypothesized that alteration in protein matrix and water acting as lubricant or plasticizer between proteins is the primary reason for increasing firmness.

In another study, it was found that high protein content and the type of protein present in matrix results in compact and dense appearance and concluded that protein content is most closely related with hardness of cheese varieties (Chen et al., 1979). The increase in hardness by high pressure was found due to gelation which is a complex process involving protein denaturation and aggregation (Ferry, 1948; Hermansson, 1997). ANOVA indicated that both treatment factors and their interaction was highly significant ($p < 0.05$). It was found that hardness of all egg components behaved in same manner (increasing hardness with increasing treatment intensity) (Table 6.14). Egg yolk (EY) showed similar pattern to that of EW but EY had 50% more increase than EW (Figure 6.2b). Egg white and egg yolk are important functional ingredients which can show gelation upon pressure processing and it can be used in preparation and texture modification of food products (Paraskevopoulos and Kiosseoglou, 1997).

Hardness of WLE also increased with increase in pressure level and treatment time but hardness values were lower than EY and higher than EW (Figure 6.2c).



(a)



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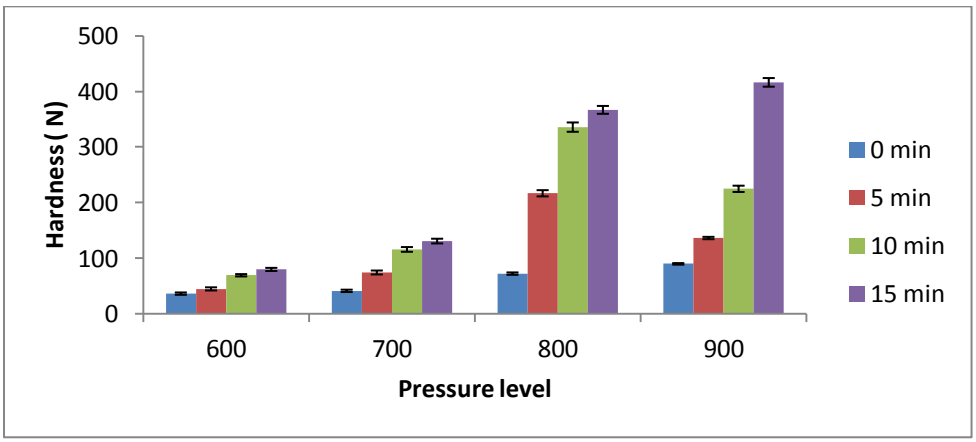


(c)

Figure 6.2: Effect of High pressure processing and treatment time on hardness of various egg components: (a) EW, (b) EY, and (c) WLE

ANOVA analysis for EY and WLE indicated that both treatment factors and their interaction were highly significant (p <0.05) (Table 6.14). It was found that pressure treated samples gave an impression similar to that of cooked egg gels but gels formed were very adhesive and elastic. In a similar study it was found that high pressure coagulated egg white gels were more adhesive and elastic than thermally treated gels (Hayashi et al., 1989). Correspondingly Carlez et al. (1995) reported that high pressure processed gels have softer structure than that of thermal treatments.

Table 6.14: ANOVA indicating significance of treatment factors and their interaction on hardness of various egg components: (a) EW, (b) EY, and (c) WLE

a) ANOVA analysis: Hardness versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	77125	25708.2	1178.60	0.000
Pressure	3	134239	44746.3	2051.41	0.000
Interaction	9	32118	3568.6	163.60	0.000
Error	16	349	21.8		
Total	31	243830			

S = 4.670 R-Sq = 99.86% R-Sq (adj) = 99.72%

b) ANOVA analysis: Hardness versus Time, Pressure

Source	DF	SS	MS	F	P
Time	3	251108	83703	4757.52	0.000
Pressure	3	397740	132580	7535.63	0.000
Interaction	9	103985	11554	656.70	0.000
Error	16	282	18		
Total	31	753115			

S = 4.194 R-Sq = 99.96% R-Sq (adj) = 99.93%

c) ANOVA analysis: Hardness versus Time, Pressure

Source	DF	SS	MS	F	P WLE
Time	3	161572	53857.4	2423.96	0.000
Pressure	3	207471	69157.1	3112.56	0.000
Interaction	9	84744	9416.0	423.79	0.000
Error	16	356	22.2		
Total	31	454143			

S = 4.714 R-Sq = 99.92% R-Sq (adj) = 99.85%

6.4.2.2 Adhesiveness

Adhesiveness is more of a surface characteristic and depends on a combined effect of adhesive and cohesive forces, and others which include viscosity and viscoelastic as well

(Adhikari et al., 2001). Adhesiveness is related to surface properties and is known as the work necessary to overcome the attractive forces between the food and surface of other materials. It is also known as negative peak beneath the base line of texture profile which depicts force required to pull out plunger from the sample (Friedman et al., 1963). In this study, it was found that adhesiveness decreased linearly with increase in pressure level and treatment time for EW (Figure 6.3a). ANOVA analysis showed that both factors affected the adhesiveness in significant ($p<0.05$) manner but the interaction effect of these two parameters showed a non-significant ($p>0.05$) role (Table 6.15). EY followed a different pattern as adhesiveness increased to its maximum value at pressure level of 800 MPa and then decreased towards end at pressure level of 900 MPa (Figure 6.3b).

Table 6.15: ANOVA analysis indicating significance of treatment factors and their interaction on adhesiveness of various egg components: (a) Egg white, (b) Egg yolk and (c) Whole liquid egg

a) Two-way ANOVA: Adhesiveness versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	2296.1	765.365	1.35	0.293
Pressure	3	1918.1	639.365	1.13	0.366
Interaction	9	5317.5	590.837	1.04	0.449
Error	16	9048.5	565.531		
Total	31	18580.2			

S = 23.78 R-Sq = 51.30% R-Sq (adj) = 5.64%

b) Two-way ANOVA: Adhesiveness versus Time and Pressure

Source	DF	SS	MS	F	P
Time	3	251.594	83.865	18.01	0.000
Pressure	3	381.094	127.031	27.28	0.000
Interaction	9	43.281	4.809	1.03	0.456
Error	16	74.500	4.656		
Total	31	750.469			

S = 2.158 R-Sq = 90.07% R-Sq (adj) = 80.77%

c) Two-way ANOVA: Adhesiveness versus Time, Pressure

Source	DF	SS	MS	F	P
Time	3	34.0930	11.3643	266.98	0.000
Pressure	3	31.1673	10.3891	244.07	0.000
Interaction	9	7.9798	0.8866	20.83	0.000
Error	16	0.6811	0.0426		
Total	31	73.9211			

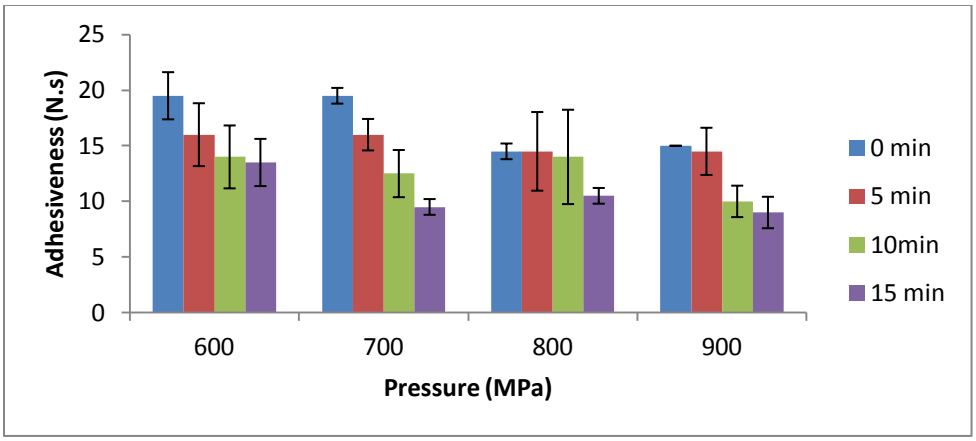
S = 0.2063 R-Sq = 99.08% R-Sq (adj) = 98.21%

ANOVA results illustrated that all treatment factors and their interaction affected adhesiveness in significant manner ($p < 0.05$). WLE followed an increasing trend, but adhesiveness value of EW was 2 fold to that of the WLE (Figure 6.3c). EY samples were more adhesive physically than EW and WLE. Egg components followed EY>EW>WLE pattern for adhesiveness, where EY demonstrated maximum hardness. Increasing pressure level from 500-900 MPa affected the adhesion properties of egg components in a very comprehensive manner. The possible reason for highest increase in egg yolk could be the higher amount of fat present in egg yolk matrix, thus increasing adhesiveness.

6.4.2.3 Springiness

Springiness is the ability of a product to spring back after deformation during first compression. Spring back is measured at down stroke of second compression, so duration time between two compressions is very important. Springiness was earlier known as elasticity.

The ability of various egg components to show springiness is very important as their use as gels and it may also influence texture and acceptability of food such as cakes, biscuits and omelets. High pressure treated EW showed small but significant ($p<0.05$) change in springiness (Figure 6.4a). ANOVA analysis showed that treatment time was less significant ($p> 0.05$) factor than pressure level ($p > 0.05$) in terms of affecting springiness (Table 6.16).



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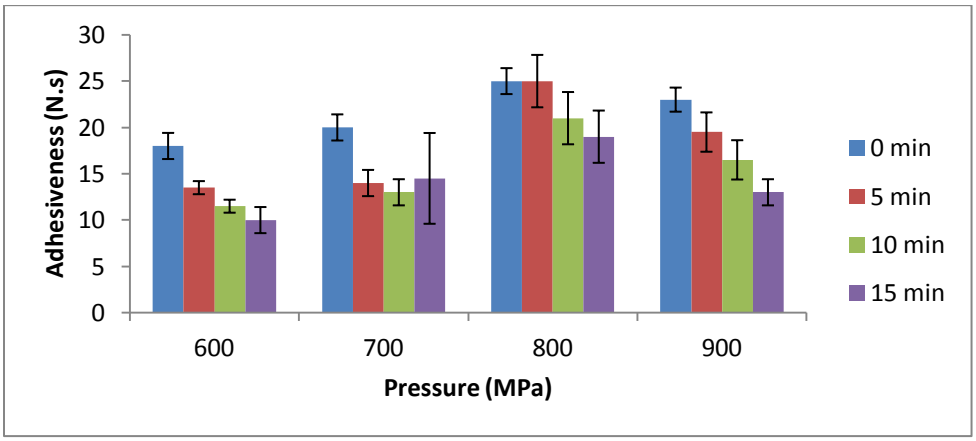

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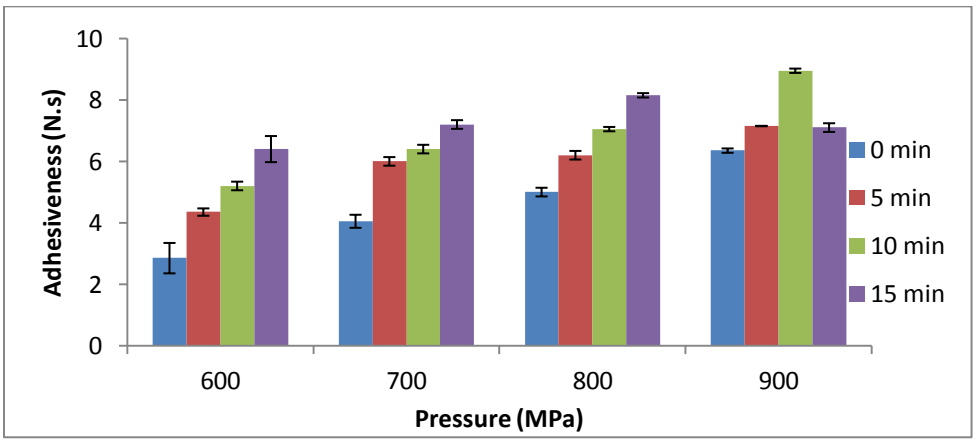
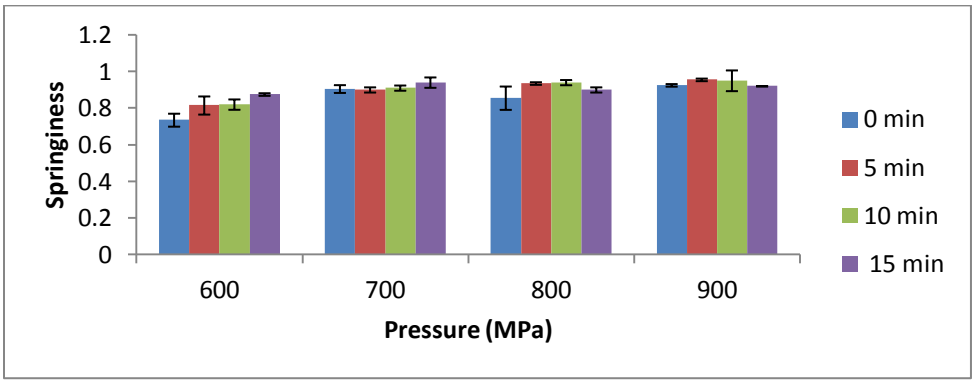


(c)

Figure 6.3: Effect of High pressure processing and treatment time on adhesiveness of various egg components: (a) EW, (b) EY, and (c) WLE



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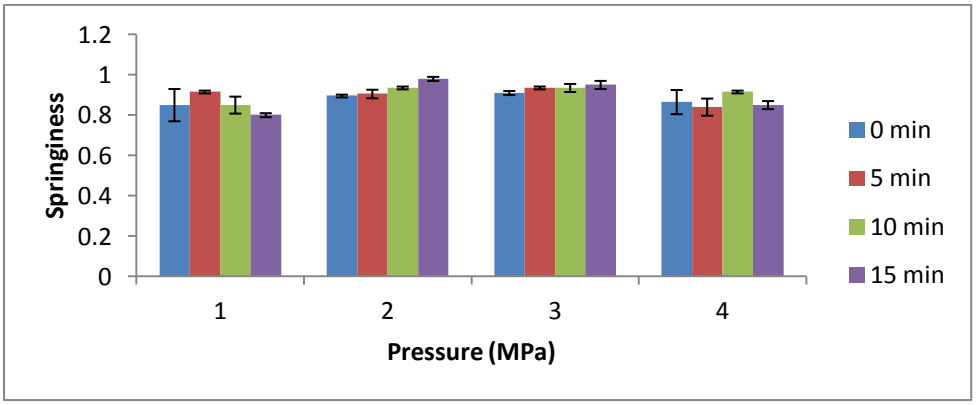

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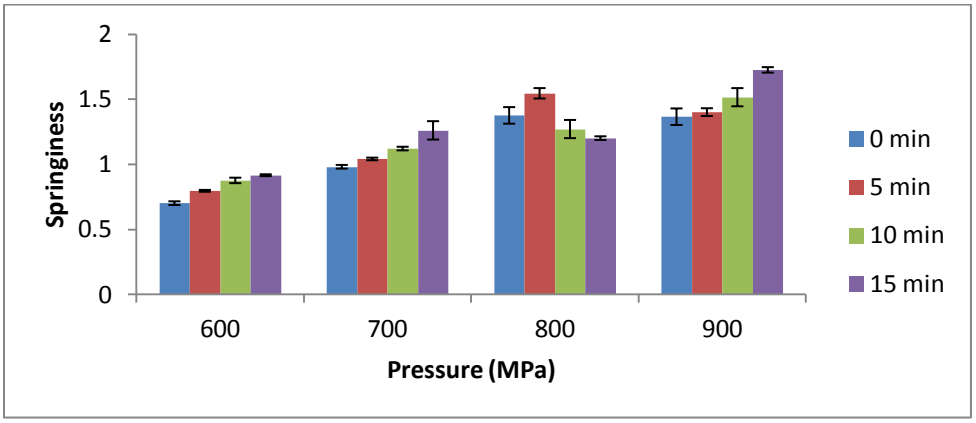


(c)

Figure 6.4: Effect of High pressure processing and treatment time on springiness of various egg components: (a) EW, (b) EY, and (c) WLE

Table 6.16: ANOVA analysis indicating significance of treatment factors and their interaction on springiness of various egg components: (a) EW, (b) EY, and (c) WLE

a)Two-way ANOVA: Springiness versus Time and Pressure EW

Source	DF	SS	MS	F	P
Time	3	0.015225	0.0050750	5.80	0.007
Pressure	3	0.074425	0.0248083	28.35	0.000
Interaction	9	0.017750	0.0019722	2.25	0.075
Error	16	0.014000	0.0008750		
Total	31	0.121400			

S = 0.02958 R-Sq = 88.47% R-Sq (adj) = 77.66%

b)Two-way ANOVA: Springiness versus Time, Pressure EY

Source	DF	SS	MS	F	P
Time	3	0.0034125	0.0011375	1.00	0.418
Pressure	3	0.0400125	0.0133375	11.73	0.000
Interaction	9	0.0269625	0.0029958	2.63	0.044
Error	16	0.0182000	0.0011375		
Total	31	0.0885875			

S = 0.03373 R-Sq = 79.46% R-Sq (adj) = 60.19

c)Two-way ANOVA: Springiness versus Time, Pressure WLE

Source	DF	SS	MS	F	P
Time	3	0.11580	0.038600	26.39	0.000
Pressure	3	2.12587	0.708625	484.53	0.000
Interaction	9	0.31953	0.035503	24.28	0.000
Error	16	0.02340	0.001463		
Total	31	2.58460			

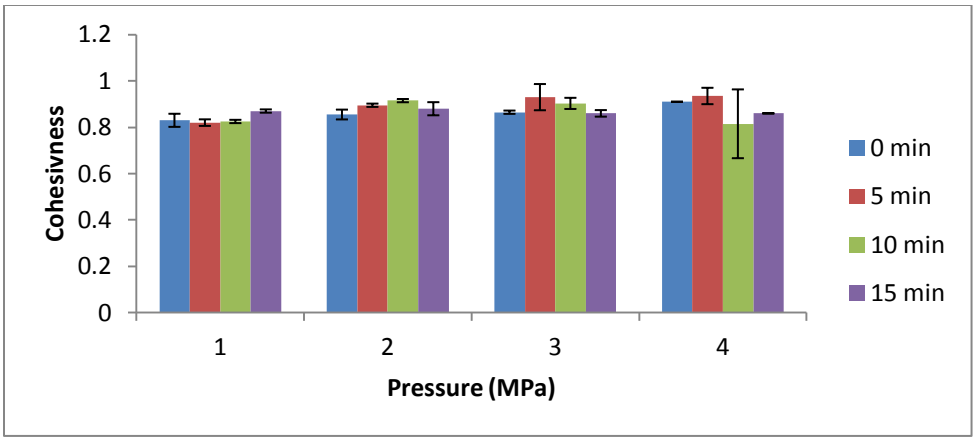
S = 0.03824 R-Sq = 99.09% R-Sq (adj) = 98.25%

Even the interaction effect of pressure and time was non-significant. On other hand for EY, springiness showed rather inconsistent pattern as it showed increase in springiness followed by decrease in its value by increasing pressure level and treatment time (Figure 6.4b). ANOVA analysis illustrated that effect of pressure was significant (p <0.05) but treatment factor was insignificant factor (p >0.05) (Table 6.16). For WLE, it was found using the ANOVA analysis that all factors and their interaction effects were highly significant (p <0.05). WLE followed a different trend than that of EW and EY as springiness increased with increasing pressure and treatment time (Figure 6.4c).

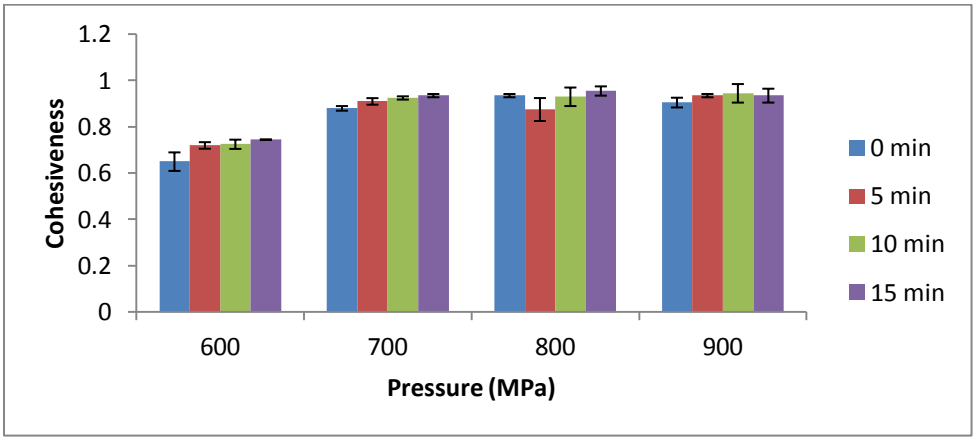
6.4.2.4 Cohesiveness

Cohesiveness is defined as ratio of area under second peak and area of first peak in a double mode compression test and it represents the internal bonds making up the body. Cohesiveness was calculated using TPA and it differed significantly in HPP treated egg samples. In case of EW, the cohesiveness showed a very small increase with increase in treatment intensity (Figure 6.5a). Even with ANOVA analysis it was found that impact of pressure, time and their interaction on cohesiveness was not significant (p >0.05) (Table 6.17). EY behaved in different manner in terms of cohesiveness characteristics than that of EW (Figure 6.5b). Increase in pressure level and treatment time both caused increase in cohesiveness, but increasing pressure level (p<0.005) was more significant than treatment time (p > 0.005) (Table 6.17). Interaction effect of pressure level and treatment time was found to be non-significant (p>0.05) (Table 6.17). Cohesiveness was not found to be showing non linear pattern in WLE, but with increasing treatment intensity, the overall behavior shown was decreasing trend in cohesiveness (Figure 6.5c). ANOVA analysis showed that effect of treatment factors and their interaction on cohesiveness was highly significant ($p \leq 0.05$) which means that varying pressure level and treatment time played a significant role in affecting cohesiveness. During compression, fracture was not calculated, so this cohesiveness may not be ideal cohesiveness. It is more likely the elastic-plastic nature of the sample which could be affected by combination of cohesiveness and adhesiveness (Kilcast and Roberts, 1998).

In a similar study, Mateos et al. (1996) found that temperature and pressure have a linear effect on cohesiveness ($p \leq 0.01$) in gels with their highest cohesiveness values occurring at low temperatures and low pressures. Heating could, perhaps, be the factor that caused cohesiveness to be significantly similar. It was found that sol-gel conversion involves protein denaturation followed by aggregation of protein molecules, eventually forming well defined highly cohesive molecular network to form solid phase (Handa et al., 1998). The protein can denature partially as high pressure treatment causes denaturation of ovotransferrin, ovalbumin and lyzozyme.



(a)



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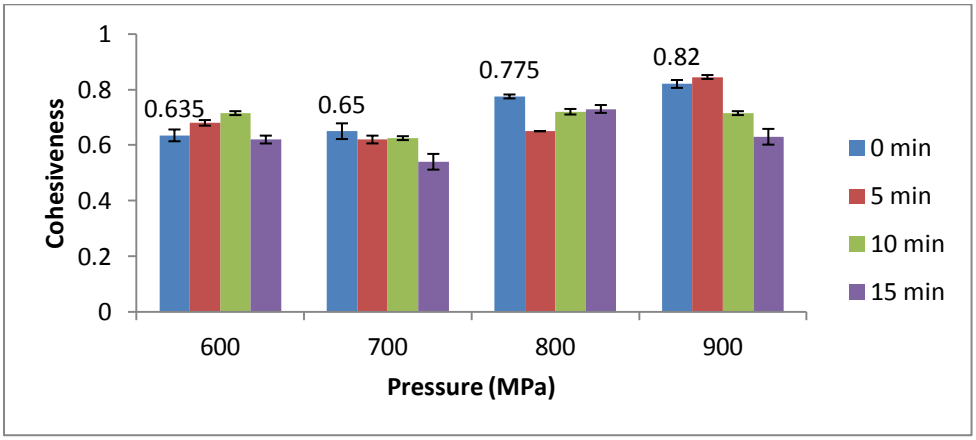

(c)

Figure 6.5: Effect of High pressure processing and treatment time on cohesiveness of various egg components: (a) EW, (b) EY, and (c) WLE

Table 6.17: ANOVA analysis indicating significance of treatment factors and their interaction on cohesiveness of various egg components: (a) EW, (b) EY, and (c) WLE

a)Two-way ANOVA: Cohesiveness versus Time and Pressure EW

Source	DF	SS	MS	F	P
Time	3	0.005204	0.0017347	0.73	0.550
Pressure	3	0.014779	0.0049263	2.07	0.145
Interaction	9	0.025457	0.0028286	1.19	0.366
Error	16	0.038128	0.0023830		
Total	31	0.083568			

S = 0.04882 R-Sq = 54.37% R-Sq (adj) = 11.60%

b)Two-way ANOVA: Cohesiveness versus Time, Pressure EY

Source	DF	SS	MS	F	P
Time	3	0.011884	0.0039615	5.13	0.011
Pressure	3	0.271134	0.0903781	117.09	0.000
Interaction	9	0.010703	0.0011892	1.54	0.216
Error	16	0.012350	0.0007719		
Total	31	0.306072			

S = 0.02778 R-Sq = 95.97% R-Sq (adj) = 92.18%

c)Two-way ANOVA: Cohesiveness versus Time, Pressure WLE

Source	DF	SS	MS	F	P
Time	3	0.036112	0.0120375	44.79	0.000
Pressure	3	0.096112	0.0320375	119.21	0.000
Interaction	9	0.063862	0.0070958	26.40	0.000
Error	16	0.004300	0.0002688		
Total	31	0.200387			

S = 0.01639 R-Sq = 97.85% R-Sq (adj) = 95.84%

High pressure can cause the denaturation and coagulation to increase the proteolysis similar to that of heat but high pressure treated gels have highly elastic and adhesive properties compared to thermally treated egg. From quality point of view, this process gives fresh and uncooked flavor in the egg gels. All egg components behave differently from each other on pressurizing due to variation in composition as egg white is rich in proteins (ovalbumin, ovotransferrin, and ovomucid); whereas egg yolk on other hand is a rich source of fat (unsaturated and saturated fatty acids). Generally, hardness is correlated to the rupture strength of the sample; springiness represents rubberiness, and cohesiveness is the degree of difficulty to break down a sample (Sanderson, 1990).

6.5 Conclusions

The effect of high pressure treatments on some physico-chemical properties on EY, EW and WLE were studied. Textural properties of all egg components were significantly affected by high pressure treatments. These processing treatments changed significantly with change in pressure level. By increasing pressure, egg components changed from liquid state to complete gel with coagulation and gelation of egg components. Egg gels were formed at high pressure level greater than 600 MPa at temperature well below that required for thermal gel formation. HPP lead to formation of full set egg gels with improved physicochemical characteristics and without any cooked flavors. Further investigation is required on microbiological aspects and functional properties of post-processed samples before implementing this study to industrial sector.

PREFACE

TO CHAPTER 7

In earlier Chapters, effect of high pressure on rheological and textural characteristics was studied. When pressure level is increased, structural changes result in modification of the functional properties. Viscoelastic studies give the detailed structural information and serves to describe the behavior of the data, ultimately serving as a prediction tool for rheology or the degree of protein unfolding and prediction of viscous and elastic modulus.

In this Chapter, functional and viscoelastic properties have been studied in correlation, to understand the changes occurring in functional properties of egg white and whole liquid egg as function of increasing pressure and treatment time.

Following manuscript has been prepared from this Chapter:

Singh A and Ramaswamy HS (2012) Functional and viscoelastic properties of high pressure treated egg components (Prepared for Submission).

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy.

CHAPTER 7

FUNCTIONAL AND VISCOELASTIC PROPERTIES OF HIGH PRESSURE TREATED EGG COMPONENTS

7.1 Abstract

Rheological and functional properties (Foam density, foam volume, turbidity and water holding capacity) in various high pressure treated egg components (egg white, and whole liquid egg) were studied as function of increasing pressure level (350-550 MPa) and holding time (5-15 min). Egg components exhibited a gradual liquid-solid gel transformation as they coagulate/denature and behave as viscoelastic fluid. Elastic (G') and viscous (G") properties of pressurized gel increased with increasing both pressure level and holding time. Egg white and WLE showed crossover of G' and G" at pressure treatment of 550 MPa for 10 min and 550 MPa for 15 min respectively. Similarly functional properties like foaming ability for egg white showed increasing trend with increase in pressure level and holding time. But on other hand, whole liquid egg were found to show non-significant changes in their functional properties. This study has provided supplementary information on pressure induced changes in the functional and rheological properties of various egg components.

7.2 Introduction

Food proteins possess wide array of functional properties and food technologists always seek proteins with versatile and broad spectrum functionality. There have been attempts to seek proteins from more economical sources, to extend traditional food and to develop food with new functional ingredients. Egg is known as a multifunctional ingredient and it fits all of above mentioned requirements. It is known for excellent foaming, gelling and emulsifying characteristics. These functional characteristics of eggs are used in food industry for variety of applications (Mine, 1995). The functional

properties of egg can be enhanced and could result in better foaming with improved stability. This enhancement will facilitate imparting desirable features including appearance, texture and consistency to wide range of food products. Improvement of functional properties can be done by modifying the structure by physical, chemical or enzymatic methods (Kato et al., 1983; Arai and Watanabe, 1988). Heat treatment can cause denaturation which results in alterations of functional properties. There have been many studies regarding alterations caused by heat in structural properties and thus in turn changes in functional properties of egg proteins (Donovan et al., 1975; Mine et al., 1990; Van de Plancken et al., 2006). Heating of egg white solution in temperature range of 50-85°C results in significant unfolding of the proteins as shown by sulfhydryl (SH) groups and higher sensitivity towards proteases. These alterations are expected to change foaming characteristics of the treated egg white solutions (Van der Plancken et al., 2007).

High pressure processing has been always investigated as an alternative to the traditional thermal processing as it can cause enhancement of functional properties of food proteins in addition to microbial (Karatas and Ahi, 1992) and enzymatic inactivation (Knorr et al., 2006). It can cause modification of quaternary and tertiary structure of protein which can lead to denaturation, aggregation and gelation (Balny and Masson, 1993; Cheftel, 1995). High pressure processing is a complex process and it involves formation of hydrogen bonds and disruption of hydrophobic bonds (Heremans, 1982). These structural changes are reversible at pressure level < 200 MPa and irreversible at pressure level >200 MPa (Lametti et al., 1998; Silva et al., 1989). Modification of protein structure by pressure treatment could lead to the enhancement of the surface properties and functionality.

Functional properties of proteins have direct relationship with structure of proteins. The HP treatment can cause number of changes in viscoelastic behavior of protein which in turn affects the functional properties (Ahmed et al., 2003). It has been reported that the addition of soybean or pea proteins to rice flour modified the mechanical properties of the rice–proteins blend dough, inducing a significant increase in the elastic modulus recorded by the oscillatory tests (Marco and Rosell, 2008). Rheological analysis

also gives useful information on the foaming and gelation. Hickson et al. (1982) measured changes in heat induced gel characteristics during cold storage of egg albumen and found that viscosity increased in correlation with functional properties like elasticity and gel strength during storage of eggs.

Elevated level of pressure causes smaller effect on surface hydrophobicity in comparison to elevated temperature. On the other hand, the decrease of exposed SH groups due to SH oxidation is enhanced by pressure (Van der Plancken et al., 2006). These observations explain the reason why pressure induced protein solubility cause lower loss of functionality as compared to heat treatment. Pressure induced structural changes in egg white proteins can also be demonstrated by the exposure of buried hydrophobic and SH groups resulting in increased flexibility (Lametti et al., 1998; Lametti and Rovere, 1999; Van der Plancken et al., 2005).

High pressure treatment cause formation and stabilization of smaller aggregates formed during denaturation of proteins, whereas heat treatment can induce formation of large, insoluble aggregates which cause turbidity in egg solutions. The foaming properties of heat and pressure treated egg white are different as mechanism of protein denaturation by heat and pressure are different (Van der Plancken et al., 2005). Pressure induced changes in protein structure will affect the foaming and functional characteristics of pressurized egg solutions. It has been known that moderate unfolding of egg white using even a simple pH induced unfolding and refolding regime could significantly improve its foaming properties (Liang and Kristinsson, 2005). Partial unfolding of egg before foaming as well as the interactions among egg proteins through disulfide and hydrophobic groups may be responsible for improvement in foaming properties.

There have been numerous studies already conducted on the pressure-induced gelation of egg white (Bridgman, 1914; Hayashi et al., 1989; Balny and Masson, 1993); but very few studies are available on the effect of pressure on the dynamic rheology (viscoelastic properties) and its correlation with foaming and other properties of treated egg.

The objective of this work is evaluate the effect of increasing pressure level (350 to 550 MPa at constant time of 5 min) and increasing treatment time (550 MPa from 5-15 min) on viscoelastic behavior of egg white and whole liquid egg and other functional properties like foaming, turbidity and water holding capacity.

7.3 Materials and methods

7.3.1 Sample preparation: Chicken eggs were purchased from local market and were broken manually to separate carefully egg white from yolk using the Harrison and Cunningham (1986) preparation method. Whole liquid eggs (WLE) were prepared by mixing egg using a manual egg beater. 10g of sample was prepared was poured into flexible 2 oz. Nasco polyethylene bags. Samples were treated immediately with high pressure processing.

Table 7.1: **Experimental design to evaluate effect of high pressure treatment**

Pressure (MPa)	350	450	550
Time(min)	5	5	5
	X	X	10
	X	X	15

7.3.2 High pressure treatment

Samples were treated using ACB France (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France) HP unit. Pressurization and depressurization rate were maintained at 4.4 MPa/s and 26 MPa/s. respectively, during high pressure treatments in addition to the selected pressure hold times. Pressure treated samples were placed in an ice box and immediately transferred to a refrigerator (4°C) until the functional properties were evaluated. Pressure come up (slightly longer at the higher pressure levels) and pressure release time were not considered in the specified holding time as shown in Table

7.1. Pressurization and depressurization rate of 4.4 MPa/s and 26 MPa/s respectively, were used during high pressure treatments. All the experiments were carried out in duplicate at room temperature conditions.

7.3.3 Functional properties

7.3.3.1 Viscoelasticity

HPP samples (2 mL) were transferred to a control stress rheometer (AR2000 series rheometer, TA Instrument, New Castle, DE, USA) and measured for changes in viscoelastic behavior. Parallel plate (6 cm diameter) geometry was used with a gap width of 1000 μm. The temperature used during measurement was 22°C which was maintained using a circulating bath and a controlled peltier system. Linear viscoelastic region (LVR) was found in initial experiments by performing the stress sweep tests at a frequency of 1 Hz in order to determine the range of linear viscoelastic response under oscillatory shear conditions. Based on LVR, oscillation stress in a frequency range between 0.1 and 10 Hz was studied. Steady shear rheology of each samples were carried out at frequency ranging from 0.1–10 s^{-1}. All rheological measurements were carried out in duplicate.

7.3.3.2 Foam density and foam stability

Each egg white sample was foamed in a cylindrical beaker (50 mL) with an egg beater. The foaming process consisted of beating 10 g of sample for 2 min at maximum speed setting. The foam density was then measured which is a measure of the thickness of the foam. It gives a view of the quantity of air incorporated in the egg white foam. This important factor tells about the aerating properties of the egg white in its food applications. The foam volume of the egg is a crucial parameter for the functional quality of the egg white. It gives us information about actual increase in egg volume due to its foam upon beating. The commercial use of egg white is highly dependent on its foam density and stability in many of its applications in the food industry.

The foam stability (FS) was taken as the quantity of liquid drained as a function of time from the completion of foaming. This measurement was taken after 60min of drainage. Samples were determined in duplicate.

7.3.3.3 Turbidity

Turbidity is a measure of haziness or cloudiness and in egg it can by caused by protein coagulation. Coagulated proteins are opaque, so they cause reduction of transmittance of light through the egg white. The absorbance of samples was measured at 650 nm using a Nova Swiss II spectrophotometer (Biochrom Ltd, Cambridge, England) at room temperature. Deionised water was used as the control. A turbidity of 0% corresponds to a totally clear solution.

7.3.3.4 Water-holding capacity

The water-holding capacity (WHC) of egg samples was evaluated using the method described by Hammershoj et al (2006) with small modification. After HPP, centrifuge tubes were filled with liquid egg (2mL) were placed in a centrifuge (Thermal IEC, IEC Multi RF, Model 120, USA) and centrifuged at 10000 × g for 30 min. WHC was calculated using following equation:

$$WHC\ (\%) = \left(\frac{A}{B}\right) \times 100 \qquad (7.1)$$

where A= weight of gel after centrifugation; B = weight of gel before centrifugation.

7.3.3.5 Statistical analysis

Mathematical and Statistical Analysis was performed on the data using analysis of variance (ANOVA) with help of Minitab 16 Statistical Software (Minitab Inc, State College, Pa, USA). ANOVA analysis was done and significance level was reported with 95% level of confidence. It was performed to evaluate effect of treatment factors (time and pressure) and their combination on the viscoelasticity and other functional properties like foaming characteristics, turbidity and water holding capacity.

7.4 Results and discussions

7.4.1 Viscoelastic characterization

Egg white

Rheological assessment is a good indicator of molecular structure and thus of end use performance of functional properties (Marin and Montfort, 1996). In case of egg, rheological analysis has been successfully applied as an indicator of molecular structure of egg and thus predictors of their functionality in the food industry (Lomakina and Mikova, 2006; Talansier et al., 2009; Van der Plancken et al., 2005).

The viscoelastic properties of the egg components were studied by dynamic oscillatory test. The mechanical spectra of all the samples showed elastic modulus (**G′**) values higher than viscous modulus (**G″**) at all the frequency range tested, which suggest a viscoelastic solid behavior of the eggs. Dynamic tests were performed to determine linear viscoelastic region for frequency dependence of the elastic and viscous modulus. Pressure treatment caused partial unfolding of egg proteins that leads to changes in structure and rheology. It was found that, increasing pressure level and treatment time caused increase in both *G'* and *G"* of egg white but elastic modulus (**G**') was significantly ($\mathbf{P} < 0.05$) higher compared to the viscous modulus (**G**") throughout the frequency range (0.1-10 Hz) confirming the increase in elasticity of egg components with increase in pressure level and treatment time.

Change in viscoelastic properties of EW and WLE was possibly due to pressure induced aggregation effect. High pressure processing facilitates random aggregation of partially denatured egg protein molecules. Linear viscoelastic properties were dependent on the pressure level and treatment time as both caused changes in rheology. This thickening effect depends upon size of aggregates formed and cause development of three dimensional structures leading to enhancement of elasticity. Effect of pressure and treatment time on elastic and viscous modulus has been shown in Figure 7.1(a-f).

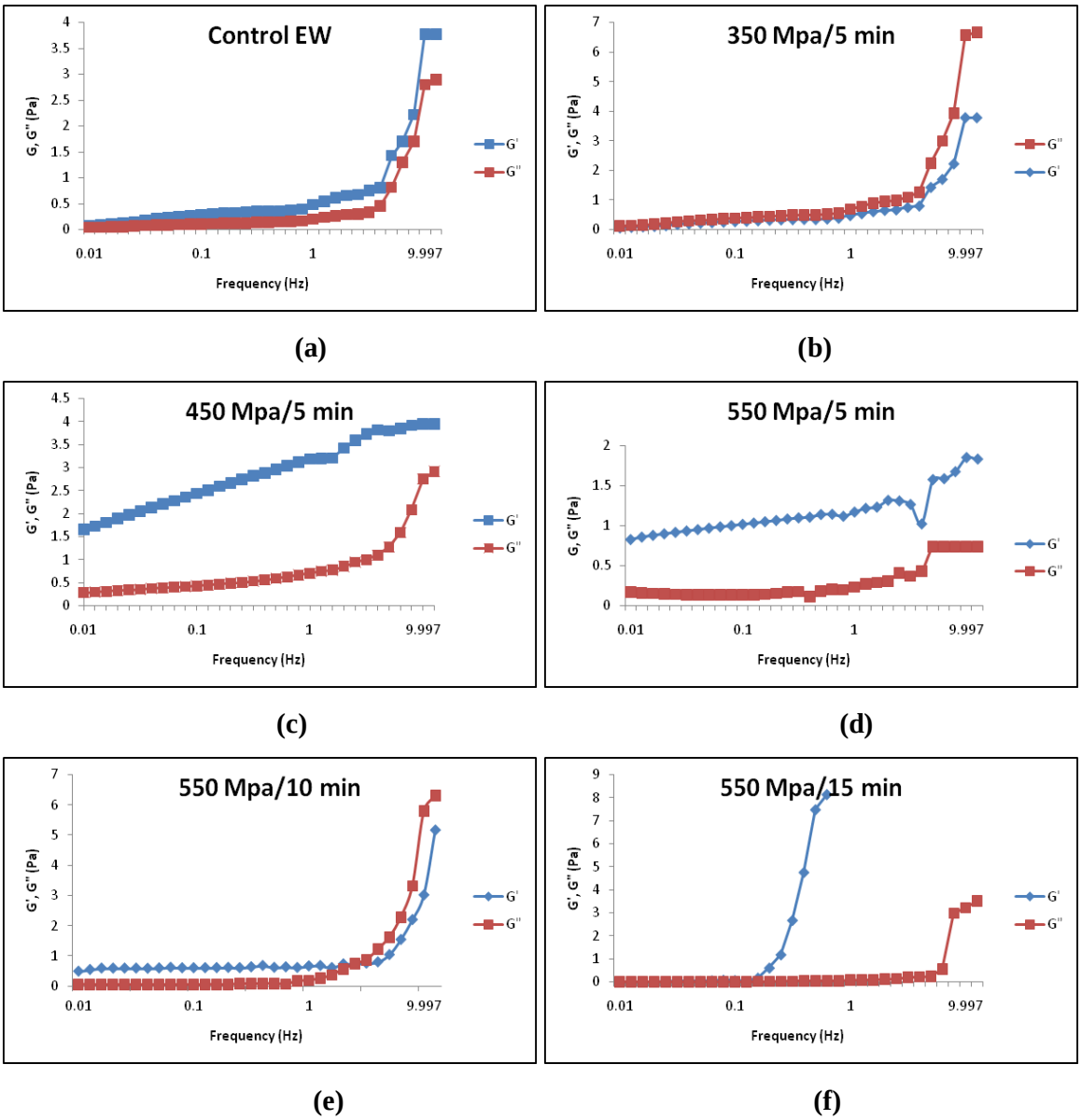

Figure 7.1: Viscoelastic parameters (G' (elastic modulus); G" (Viscous modulus)) graph of egg white (EW) as affected by different pressure level and treatment time (a) G' and G" graph of untreated egg at atmospheric pressure (0.1 MPa). (b) G' and G" graph after pressure treatment of 350 MPa for 5 min (c) G' and G" graph after pressure treatment of 450 MPa for 5 min (d) G' and G" graph after pressure treatment of 550 MPa for 5 min (e) G' and G" graph after pressure treatment of 550 MPa for 10 min (f) G' and G" graph after pressure treatment of 550 MPa for 15 min

Elastic modulus (*G'*) and viscous modulus (*G"*) tend to increase very rapidly by increasing pressure from 350 to 550 MPa for treatment time of 5 minutes but increase in *G'* was much higher than *G* (Figure 7.1b-d). Similarly increase in treatment time (5 to 15 min) at pressure level (550 MPa) caused increase in viscous modulus (*G"*) in similar fashion as of elastic modulus (*G'*) (Figure 7.1d-f). Pressure treatment of 550 MPa for 10 min caused the greater increase of *G"* (viscous modulus) as compared to *G'* (elastic modulus) resulting in cross over of *G'* and *G"* (Figure 7.1e). This crossover is referred to as gelling point where gel is formed.

This observable fact could be explained by physical proximity of the egg white protein molecules which favors intermolecular interactions leading to formation of elastic gel structure. Ahmed et al. (2005) found important correlation between protein concentration and viscoelastic behavior of pressure induced soy protein isolate gels. He found that HP treatment at higher levels caused a continuous increase of both *G'* and *G"* in 15% soy protein concentrate. Delta degree was used to evaluate the shift from liquid like behavior to solid behavior. Delta degrees is the tangent of the phase angle – the ratio of viscous modulus to elastic modulus and a useful quantifier of the presence and extent of elasticity in a fluid Delta degree value of less than 45 degrees defines solid-like behavior, while Delta degree value of more than 45 degrees interprets liquid like behavior (Alvarez et al., 2008). Plotting the same data in terms of delta degrees versus frequency (Figure 7.2), suggested that all high-pressure treatment induced changes in the Delta degrees; however the largest change occurred at 550MPa/5 min (Figure 7.2). Figure 7.2 shows that increase in pressure level from 350 to 550 with same treatment time (5 min) did not caused any significant changes in delta degrees. Here delta degree was around 40 indicating solid like consistency. But on other hand, if treatment time is increased from 5-15 minutes at 550 MPa, there were significant changes in delta degrees. At this point, delta degree of > 45 indicates higher level of denaturation causing oozing out of water resulting in liquid like behavior. These differences could be the result of increased solubility under pressure for precipitated samples and the rearrangement of molecular networks, which, depending on the specific conditions, could result in an increase or decrease of both elastic and viscous moduli.

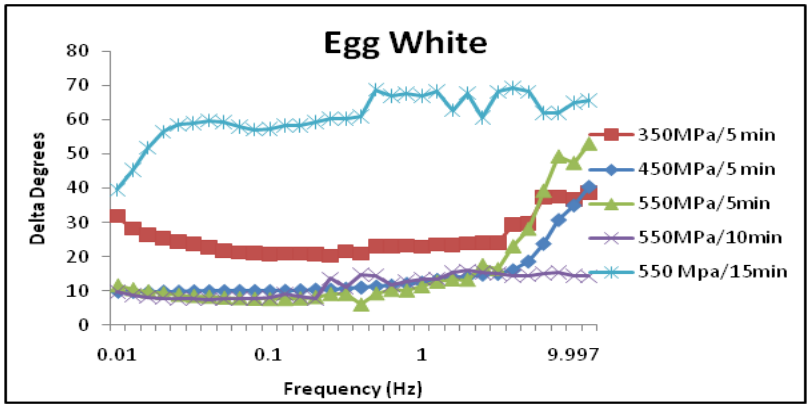

Figure 7.2: Delta degrees of different egg white (EW) subjected to various pressure treatments

Whole liquid egg

Whole liquid egg (WLE) showed similar changes as that of egg white, but extent of changes in both viscous (*G"*) and elastic modulus (*G'*) was small. Increase in pressure level from 350 to 550 MPa for treatment time of 5 min increased both *G'* and *G"* (Figure 7.3b-d). Pressure treatment of 550 MPa for treatment time of 15 min produced crossover of both *G'* and *G"*. Elastic behavior increased few folds with increase in pressure level, though there was no systematic pattern observed. As the pressure level increased, *G'* increased between 0.1 and 10 Hz with respect to control.

Whole liquid egg (WLE) showed similar changes as that of egg white, but extent of changes in both viscous (*G"*) and elastic modulus (*G'*) was small. Increase in pressure level from 350 to 550 MPa for treatment time of 5 min increased both *G'* and *G"* (Figure 7.3b-d). Pressure treatment of 550 MPa for treatment time of 15 min produced cross of both *G'* and *G"*. Elastic behavior increases few folds with increase in pressure level, though there was no systematic pattern observed. As the pressure level increased, *G'* increased between 0.1 and 10 Hz with respect to control.

Pressure treatments caused changes to viscous behavior in comparsion to that of control WLE sample in a non-systematic way. With increase in pressure level from 350 to 550 MPa, it showed increase in both *G'* and *G"* but when treatment time was increased from 5 to 15 min at pressure level of 550 MPa, there was dramatic rise in viscous modulus (*G"*) causing the cross of *G'*and *G"* leading to gel point (Figure 7.3d-f).

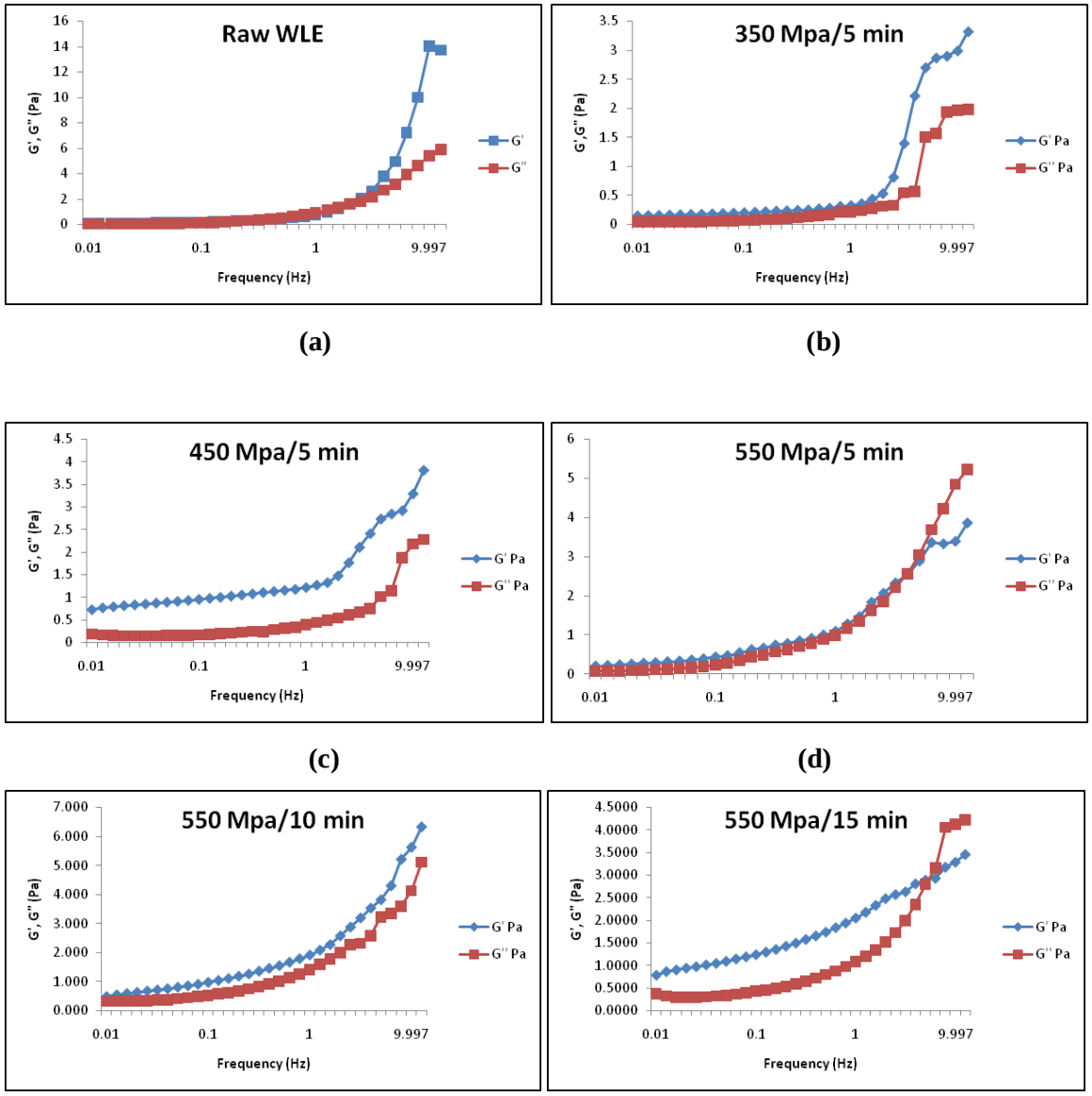

Figure 7.3: Viscoelastic parameters (G'; G") of whole liquid egg (WLE) as affected by different pressure level and treatment time (a) G' and G" graph of untreated whole liquid egg at atmospheric pressure (0.1 MPa). (b) G' and G" graph after pressure treatment of 350 MPa for 5 min (c) G' and G" graph after pressure treatment of 450 MPa for 5 min (d) G' and G" graph after pressure treatment of 550 MPa for 5 min (e) G' and G" graph after pressure treatment of 550 MPa for 10 min (f) G' and G" graph after pressure treatment of 550 MPa for 15 min

Pressure treatments increased the solubility for precipitated samples which causes the rearrangement of molecular networks, depending on the specific conditions, could result in an increase or decrease of both elastic and viscous moduli (Ahmed et al., 2007). Delta degree of WLE showed major changes as pressure level and treatment time increased. For raw WLE, Delta degrees was 53 (more than 45) showing liquid like characteristics and after pressurization at 350 MPa for 5 min, it changed to 23 showing solid like behavior due to gelation and increase in viscosity due to pressurization. With increase in pressure level from 350 to 550 MPa for treatment time of 5 min, Delta degrees changed from 23 to 53, changing WLE from solid to liquid state. When treatment time was increased from 5 to 15 min at pressure level (550 MPa), it caused minimal increase from 53 to 64 (Figure 7.4).

HP treatments are known to cause changes in Sulfhydryl (SH) and disulfide (SS) bonds, so it is expected that both SH and SS groups undergo changes affecting the functional properties of egg. It was reported that increase in free SH residue can be caused by reduction of SS bonds. Thus breaking and formation of bonds could be the reason for unsystematic variations of dynamic moduli after pressurization (Kajiyama et al., 1995).

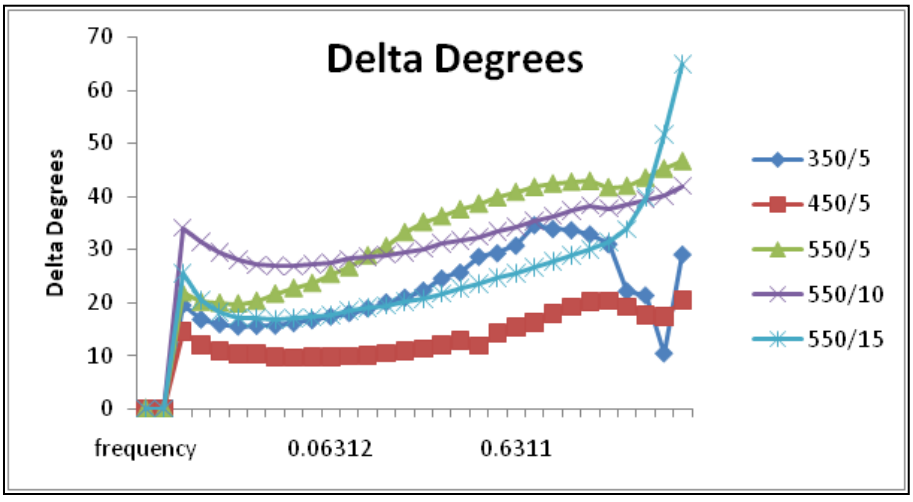

Figure 7.4: Delta degrees of different WLE subjected to various pressure treatments

7.4.2 Foam density and foam stability

Eggs are known to have excellent foaming properties. These properties are determined by the ability to rapidly adsorb air at the air-liquid interface during whipping, and by its ability to form a cohesive viscoelastic film by way of intermolecular interactions (Mine, 1995). High pressure and treatment time are known as significant parameters affecting the foaming behavior of food proteins (Halling, 1981). In this study, the influence of the increase in intensity of pressure and treatment time on the foam density and foam stability of egg white and whole liquid egg components was studied (Figure 7.5). It was found that increase in pressure level from 350 to 550 MPa showed a significant increase (p <0.05) in foam density of egg white, but on other hand when treatment time was increased from 5 to 15 min at pressure level of 550 MPa, it followed a decreasing pattern (Figure 7.5). Similarly, for whole liquid egg, increasing pressure level from 350 to 550 MPa for constant treatment time of 5 min, there was small but significant (p <0.05) increase in foam density, but on other hand when treatment time was increased from 5 to 15 min at constant pressure level (550 MPa), foam density showed a decreasing pattern.

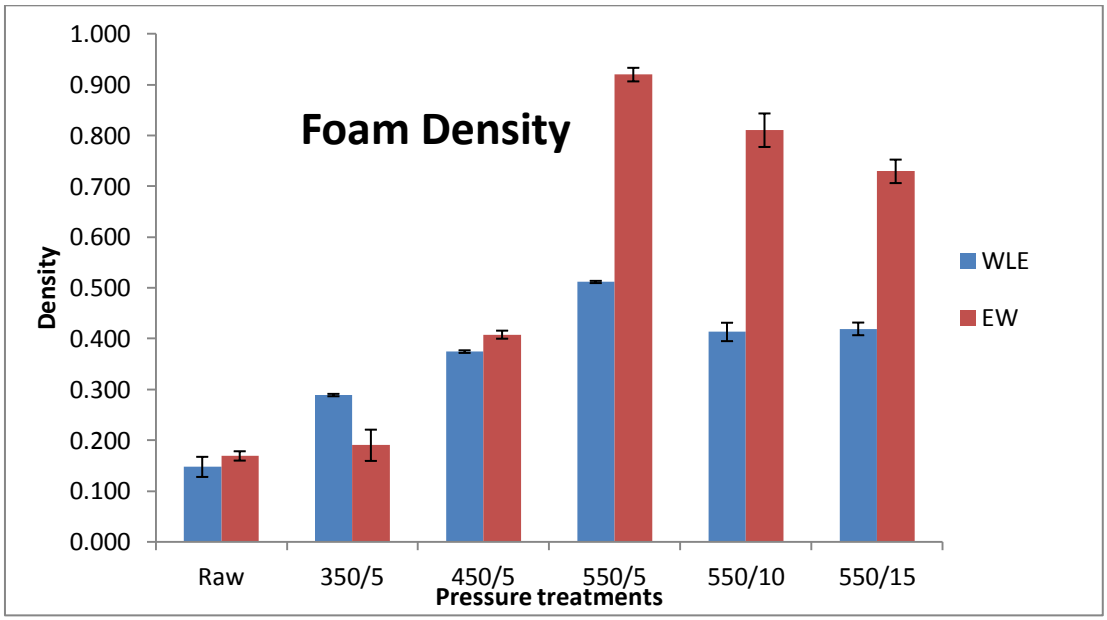

Figure 7.5: **Foam density of untreated and pressure treated egg white and whole liquid eggs**

Foam stability was found to be significantly affected by both increasing pressure level and treatment time. By increasing pressure level (350 to 550 MPa) and keeping the treatment time constant (5 min), foam stability increased up to its maximum level at pressure treatment of 450 MPa/5 min, and then it followed a decreasing pattern (Figure 7.6). On other hand, increase in treatment time (5 to 15 min) with constant pressure level (550 MPa), foam stability decreased significantly (p <0.05).

Pressure treatment of 550 MPa for 5 min was found to have almost same foam stability to that of raw whole liquid egg (Figure 7.6). The main reason in increase of foam density could be due to extensive unfolding of protein chains causing subsequent intermolecular interactions after pressure treatment (Mozhaev, 1996). Pressure applications can disrupt hydrophobic interactions and ionic bonds resulting in protein molecules being more flexible to adsorb at faster rate (Dickinson and James, 1998). Thus increase in foam density and loss of solubility can be explained by aggregation, which is caused by association of unfolded proteins through hydrophobic interactions and facilitated due to the absence of repulsive forces near iso-electric point of egg proteins (Molina et al., 2001).

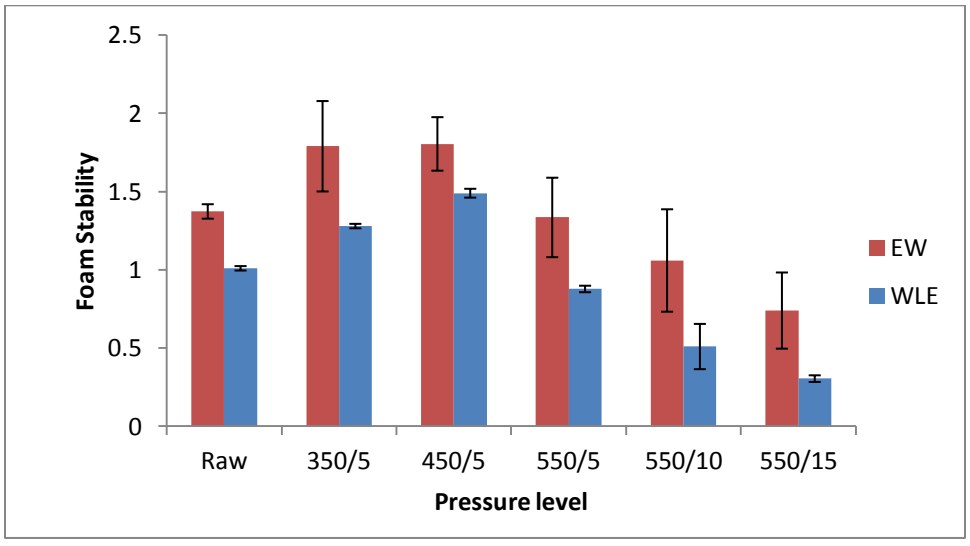

Figure 7.6: **Foam stability of untreated and pressure treated egg white and whole liquid eggs**

While evaluating these foaming properties of HP treated EW and WLE solutions, it is important to keep in mind that during foam formation, the proteins undergo additional denaturation at the interface. Conformational changes in ovalbumin at the air–water interface lead to the formation of new intermolecular disulfide bonds due to oxidation of exposed Sulfhydryl groups (Kitabatake and Doi, 1987). These pressure induced changes in the egg proteins can facilitate or counteract these changes, and thus enhance or diminish their foaming properties depending on pressure level and treatment time used.

7.4.3 Turbidity

Turbidity is indicator of denaturation and other changes occurring in egg white. In EW, Increase in pressure level (350-550 MPa) at constant time (5 min) caused any significant changes in turbidity level. Pressure treatment of 450 MPa for 5 min had same turbidity as that of raw egg white. On other hand, increase in treatment time (5 to 15 min) with constant pressure level (550 MPa) did not cause any significant change in turbidity level (Figure 7.7).

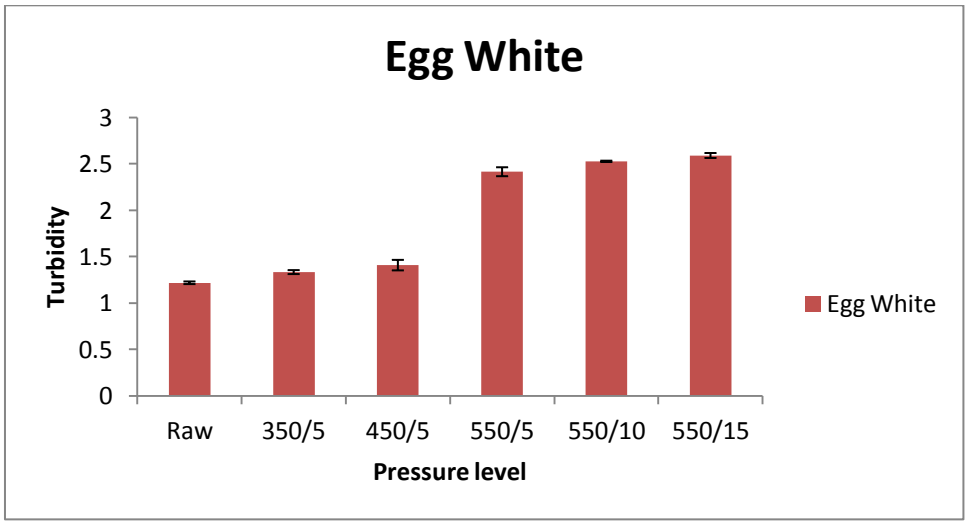

Figure 7.7: **Turbidity of untreated and pressure treated egg white**

7.4.4 Water-holding capacity (WHC)

Increase in pressure level from 350 to 550 MPa for 5 min caused significant (p <0.05) increase in water holding capacity of EW. WHC followed a decreasing pattern, when treatment time was increased from 5 to 15 min at constant pressure of 550 MPa. For WLE, increasing pressure level at constant treatment time caused linear increase in water holding capacity but on other hand, increase in treatment time from 5 to 15 min at constant pressure level (550 MPa) caused linear decrease in water holding capacity (Figure 7.8). It has been already found that high-pressure treatment helps reduce water loss, probably because of disaggregation and unfolding of protein and pH of the mixture (Cheftel and Culioli, 1997).

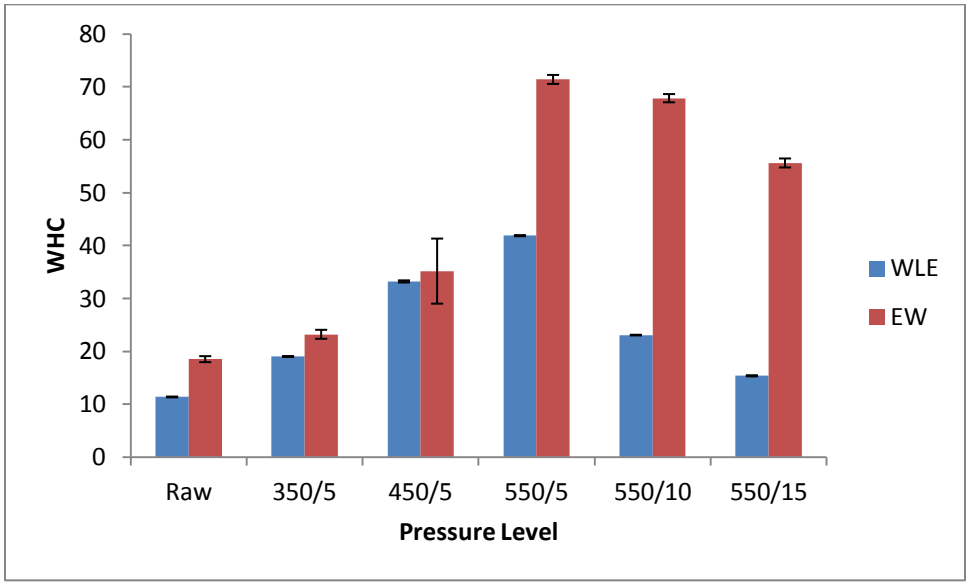

Figure 7.8: **Water holding capacity (WHC %) of untreated and pressure treated egg white and whole liquid eggs**

HPP causes destabilization of non-covalent interactions (negative volume change associated with chemical bond breakage is enhanced by pressure) (Pollard and Weller, 1966). Globular proteins are unfolded upon pressure processing and may show increase

in water holding capacity. It was found that quaternary structure of proteins is normally held together by non covalent bonds, are separated by applying low pressure (>150 MPa) causing partial denaturation of proteins (Rademacher and Kessler, 1996). Tertiary structure of egg white protein is a three dimensional configuration as a consequence of non covalent interactions between side chains of amino acids (Johnston et al., 1992). Surface hydrophobicity has been known to increase following pressure release. Higher level of pressure and pressure holding time can cause higher exposure of hydrophobic groups (Balny and Masson, 1993). Therefore, it can be concluded that a pressure treatment, can cause a decrease in volume resulting from water-binding around charged groups, water structuring around newly solvent-exposed a polar groups (hydrophobic hydration), and solvation of polar groups through hydrogen bonding.

7.5 Conclusions

The effect of high pressure processing on viscoelastic and functional value of egg white and whole liquid egg was evaluated. It was found that high pressure causes increase in viscosity of the egg samples due to denaturation and aggregation.. High pressure treatment caused enhancement of foaming and other functional properties. It was found that treatment of 550 MPa for 5 min gave maximum value of foaming ability and water holding capacity. Pressure treatments using higher treatment time lead to diminishing of functional value. Viscosity of pressure treated egg white was found to be increasing as indicated by turbidity data. This shows that high pressure processing when used in proper combination with time-temperature can be used to enhance functionality of egg and egg products.

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PREFACE
TO CHAPTER 8

So far we have discussed modification of functional properties caused due to structural changes in egg components. Functional modification is due to unfolding of protein molecules which can expose the hidden peptides responsible for antioxidant activity. This way high pressure processing could cause enhancement of antioxidant activity due to partial unfolding of egg white.

In this Chapter, effect of high pressure as a function of pressure level and treatment time has been studied. HP was used in combination with trypsin enzyme to study degree of hydrolysis and antioxidant activity. HP results in partial unfolding which enhances the action of trypsin enzyme causing increase in degree of hydrolysis and antioxidant activity.

Part of this work has been presented in the annual meeting of Institute of food technologists, Las Vegas, USA, June 2011.

Singh A, Singh P and Ramaswamy HS (2012) HP effect on trypsin hydrolysis of egg white proteins (Prepared for Submission).

The experimental work and data analysis were carried out by the candidate under the supervision of Dr. H. S. Ramaswamy.

CHAPTER 8

EFFECT OF HIGH PRESSURE PROCESSING ON TRYPSIN HYDROLYSIS AND ANTIOXIDANT PROPERTIES OF EGG WHITE PROTEINS

8.1 Abstract

Effect of high pressure processing (HPP) [pressure level (350-550MPa) and treatment time (5-15 min)] was explored on degree of hydrolysis (DH) and antioxidant activity (AA) of egg white protein (EWP). For the hydrolysis, egg-white proteins were subjected to enzymatic (trypsin) hydrolysis process up to 120 min after which the enzyme activity was terminated. The hydrolysed products from EWP (EWH) and HP treated EWP (EWH-HP) were then evaluated for antioxidant activity. HP treatment caused substantial increase in DH of EWP, ranging from 2.78%±0.022 (untreated EWP) to 11.3%±0.03 (HP treated EWP). HP treatment had an emphatic effect on AA of EWP (concentration 10 % w/v) with AA increasing from 9.34% for untreated EWP to 19% after the 5 min treatment at 350 MPa and 25% after the same treatment at 550 MPa. With changing time from 5 to 15 min (at 550 MPa), the AA values were 9.34% for untreated EWP and 25% and 30.7%, respectively. Similar trends were observed for both DH and AA at other concentrations of EWP (2.5, 5.0 and 7.5). SDS PAGE was used for confirmation of effect HP on the hydrolysis of egg proteins. A significant rise in AA was noticed with an increase in both treatment time (5-15min) at constant pressure (550MPa) as well as at higher treatment pressures (350-550 MPa) with a given treatment time.

8.2 Introduction

Egg is a rich source of bioactive compounds which are of prime importance in human diet. It supplies all essential amino acids, vitamins and minerals required for growth and maintenance of the human body. It contains protein of high biological value as compared to other dietary proteins and they are used in many food product preparations to provide functionality. Egg proteins are also being recognized for their

antioxidant properties (Sakanaka et al., 2004). Antioxidant activity of proteins is mainly due to their ability to inactivate reactive oxygen species, scavenging of free radicals and reducing hydro-peroxides. Egg proteins have ability to inhibit lipid oxidation in food products with their antioxidant properties. Lipid oxidation results in off flavor formation, changes in color and reduction in nutritional quality of food products; therefore it is a serious concern in the food industry (Li, 2002). Control of lipid oxidation by antioxidants naturally present in food has been studied by various researchers (Taylor and Richardson, 1980). Antioxidant properties of proteins from sources like soy have been reported (Park et al., 2005). Enzyme derived soy peptides act as natural antioxidants against lipid peroxidation and the primary structure of those peptides is critical for their antioxidant activity (Chen et al., 1996). On the other hand, natural antioxidants and cellular anti-oxidative enzymes are also known for controlling harmful radicals formed in human body during oxidative metabolisms leading to chronic diseases like atherosclerosis, arthritis, dystrophy, cataracts and ageing (Xu, 2008). With increasing awareness towards high quality natural foods, the negative image of synthetic antioxidants has lead to increased use of natural antioxidants.

Several studies have shown the ability of food proteins to act as antioxidants. Lipid oxidation in oil in water emulsions was inhibited by whey protein concentrate (Elias et al., 2008). Soy protein had inhibitory action on oxidation of ethyl esters of eicosapentaenoic acid (EPA) dried in maltodextrin-stabilized, free dried emulsion powder (Park et al., 2005). Food protein has a major potential to act as an antioxidant upon their hydrolysis as it causes breakdown of protein structure and releases low molecular peptides which in turn act as antioxidants. Egg hydrolysates have been found to prevent lipid oxidation in different muscle foods such as beef, pork and tuna (Sakanaka and Tachibana, 2006). Hydrolysis of proteins can be done using enzymes or other methods like high pressure processing. Hydrolysates have higher intestinal absorption due to increase in peptide content and solubility. So this way high pressure processing (HPP) could act as a useful tool in boosting the hydrolysis process.

HPP is a well recognized novel non-thermal food processing method which is used for improving functional and nutritional importance of food (Ahmed et al., 2007). It is known for causing minimal degradation of nutritionally important compounds like vitamins, proteins, flavoring agents, thus improving overall quality of food. This can result in protein conformation changes leading to modification of secondary, tertiary and quaternary structure of macromolecules while not affecting other molecules responsible for nutritional and sensory properties (Cheftel, 1992). The tertiary and secondary structures of proteins can be significantly modified at pressures above 200 MPa. Final changes in conformation after HPP denaturation can cause full or partial unfolding of polypeptide structure which eventually results in exposure of peptides that are responsible for antioxidant activity (Messens et al., 1997). Hence, it can result in an increased antioxidant activity of proteins.

There has not been much work done on influence of HPP on health promoting antioxidant activity of egg proteins. Pressure induced denaturation of egg proteins is a complex process involving disruption of non-covalent interactions within protein molecules and leading to subsequent reformation of intra-molecular and inter-molecular bonds within protein molecules. HPP has been used to extend degree of hydrolysis of egg white protein and in turn improve antioxidant activity of egg protein by causing unfolding of protein chains and thus release of possible peptides responsible for antioxidant activity (Zhu et al., 2006). Some studies have been carried out on the effect of HPP on protein functionality (Ahmed and Ramaswamy, 2003), but connecting these results to improve digestibility and antioxidant activity of proteins has not been fully explored. Therefore, the objectives of this study were 1) to first investigate the degree of enzyme (trypsin) hydrolysis and antioxidant activity of egg white protein to provide base data; 2) to evaluate the influence of different HP treatments (with pressure and treatment time as variables) on egg white protein hydrolysis and the resulting antioxidant activity; and 3) use SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) for confirmation of hydrolysis.

8.3 Materials and methods

8.3.1 Sample preparation

Egg white protein (EWP), and all major chemicals including DPPH, o-Phthaldialdehyde and enzyme trypsin (E.C. 3.4.21.4, 10^4 units /mg) were procured from Sigma Corporation, (St. Louis, MO, USA). EWP (2.5, 5.0, 7.5 or 10 mg/mL) was prepared using 50 mM sodium phosphate buffer (pH 7.0). These were subjected to different HP treatments by keeping the untreated sample as control. Both treated and control samples were then subjected to hydrolysis using enzyme trypsin. The degree of hydrolysis was evaluated every 15 min up to 120 min at which time the enzyme was inactivated to stop the hydrolysis. These final hydrolyzed samples were then tested for antioxidant activity.

8.3.2 High pressure treatment

High pressure treatments were carried out in the high pressure equipment ACIP 6500/5/12VB (ACB Pressure Systems, Nantes, France) with a cylindrical pressure chamber with a volumetric capacity of 5 liter. Egg white proteins (EWP) samples with selected concentration levels (2.5-10 mg/mL) were packed in low density 2 oz. polyethylene bags (Whirl Pak$^{(R)}$, USA) (10 mL sample per pouch) and sealed. Pure water was used as pressurization medium in the HP unit. Test samples were pressure treated under different conditions as shown in Table 8.1. Pressurization and depressurization rate were maintained at 4.4 MPa/s and 26 MPa/s. respectively, during high pressure treatments in addition to the selected pressure hold times. Pressure come up (slightly longer at the higher pressure levels) and pressure release time were not considered in the specified holding time as shown in Table 8.1. All the experiments were carried out in duplicate. Pressure treated samples were placed in an ice box and immediately transferred to a refrigerator (4°C) until the tests were carried out for the degree of hydrolysis (DH) and antioxidant activity (AA).

Table 8.1: Experimental design to evaluate effect of high pressure treatment

Pressure (MPa)	350	450	550
Time(min)	5	5	5
	X	X	10
	X	X	15

8.3.3 Measurement of the degree of hydrolysis (DH)

The DH was estimated by quantification of cleaved peptide bonds as assessed by the O-Phthaldialdehyde (OPA) spectrophotometric assay. OPA method was slightly for measuring degree of hydrolysis (Church et al., 1983). Briefly the employed method consisted of mixing 25 mL of sodium tetra borate (100 mM), 2.5 mL SDS (20% w/w), 40 mg OPA (dissolved in 1 mL methanol) and 100 µL β- mercapto-ethanol. This mixture was made up to a final volume of 50 mL using distilled water to make the OPA reagent. For the assay, 25 µL of sample (EWP or EWH) was added to 2 mL OPA reagent and solution was mixed well. The solution was incubated for 2 min at room temperature and absorbance was measured at 340 nm using a Nova Swiss II spectrophotometer (Biochrom Ltd, Cambridge, England)

The degree of hydrolysis (DH) was computed using the following Equation:

$$\text{Degree of Hydrolysis (DH)} = \frac{(Molecular\ weight \times \Delta_{340nm}) \times 100}{d.e.p} \quad \textbf{(8.1)}$$

where Δ_{340nm} = absorbance at 340 nm

d =dilution factor

e = average molar absorption of amino acid (6000 M^{-1} cm^{-1})

p = protein concentration

8.3.4 Antioxidant activity

DPPH radical scavenging assay method was used for measuring AA in untreated and HP treated EWP before and after enzyme hydrolysis based on the method suggested by Shimada et al. (1992). Protein sample (2 mL) at different concentrations (2.5, 5, 7.5 or 10 mg/mL) was added to 2 mL DPPH (0.1mM) dissolved in 95% ethanol. Solution was mixed properly and incubated for 30 min at room temperature (in dark). The absorbance of mixture was measured at 517 nm. Antioxidant activity was measured using Eq. 8.2.

$$\text{Antioxidant Activity (AA)} = \frac{(Blank_{Absorbance} - Sample_{Absorbance}) \times 100}{Blank_{Absorbance}} \quad \textbf{(8.2)}$$

8.3.5 SDS-PAGE

Electrophoresis is an efficient separation process used mainly for determination of molecular weight of proteins using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Running buffer consisted of tris-base glycine buffer with 1% SDS. Electrophoresis run time varied between 1.5-2 hr and it was terminated when tracking dye front reached bottom of gel. It was performed as confirmation of the degree of hydrolysis achieved through enzyme initiated hydrolysis or enhanced through high pressure treatment. At end of the electrophoresis process, gels were removed from glass plates and immersed in Coomassie Blue R-250 (0.1 % w/v) overnight for staining. Destaining solution (20% methanol and 10% acetic acid) was used for destaining of gels until background color was removed (Balny et al., 1989). The desired gels were stored in 7 mL acetic acid/100 mL until they were photographed.

8.3.6 Statistical analysis

Generalized linear model was used for analysis of variance using the SAS (SAS Institute Inc. Cary, NC, USA) software package (Version 9.2). Analysis of variance was used to determine the significance of the effect of interaction between high pressure level and treatment time on DH and AA. Statistical significance for differences was tested at 5% probability level (p <0.05)

8.4 Results and discussions

8.4.1 Effect of HP treatment on the degree of hydrolysis

Effect of trypsin hydrolysis on DH of egg white protein before and after high pressure processing was evaluated. Trypsin hydrolysis on egg white was evaluated for 120 min by measuring degree of hydrolysis (DH). EWP without the addition of the trypsin enzyme showed only a small increase in the degree of hydrolysis (DH) during the entire time period of hydrolysis i.e. 120 min (Table 8.2). In case of EWP treated with enzyme trypsin, it was observed that DH increased rather steeply up to 90 min and then decreased slightly towards end. Maximum DH value of EWP without typsin hydrolysis and with typsin hydrolysis was found to be 2.78% and 6.35%, respectively after 90 min (Table 8.2). The results revealed that trypsin plays an important role in increasing DH. These results are in agreement with results obtained on hydrolysis of cellulose (Sattler et al., 1989). In food industry, enzymes are widely used to modify proteins by hydrolysis in order to improve their nutritional and functional properties. Trypsin is an alkaline protease which is frequently used to enhance the degree of protein hydrolysis (Jost and Monti, 1977). It is naturally produced in human intestine and is responsible for hydrolysis of proteins, thus increasing their digestibility. Hence the in-vitro hydrolysis gives an indication of its digestion in the human system.

High pressure processing is also recognized for altering the functionality of proteins and increase in degree of hydrolysis of food proteins (Hayashi et al., 1987). Use of high pressure treatment for enhancing DH of EWP was the main focus of this study. In order to minimize the number of experiments and at same time get the influence trend, HP treatments were limited to various pressure levels (350-550 MPa) with a specific holding time of 5 min, and increasing holding time (5-15 min) at a specific pressure level of 550 MPa. High pressure treatment of EWP was followed by trypsin hydrolysis since preliminary tests indicated only small increase in the hydrolysis of protein just by the pressure treatment itself. Degree of hydrolysis (DH) was found to increase with an increase in both pressure level and pressure holding time, each complementing the other, when compared to protein samples subjected to similar hydrolysis without HP treatment.

Table 8.2: Effect of hydrolysis of EWP with and without addition of trypsin enzyme

Time (min)	EWP Untreated	EWP enzyme hydrolyzed
0	0.93± 0.001^{g}	2.25±0.0^{i}
15	1.23± 0.0^{f}	2.71±0.014^{h}
30	1.53±0.0^{e}	3.30±0.007^{g}
45	1.66±0.21^{d}	3.86±0.018^{f}
60	1.50±0.11^{e}	5.03±0.0^{e}
75	1.93±0.002^{b}	6.01±0.0^{d}
90	2.78±0.022^{a}	6.35±0.0^{a}
105	1.72± 0.02^{c}	6.19±0.021^{b}
120	1.50±0.002^{e}	6.07±0.02^{c}

Means within columns with same letters are not significantly different *($p>0.05$)*

Highest rate of hydrolysis was achieved for protein samples treated at 550 MPa for 15 min (maximum pressure and highest treatment time employed in the study) with 2 fold increase observed in DH over EWP without treatment (Table 8.3). The least increase in HP treated samples was associated with the lowest pressure and shortest treatment time (350 MPa, 5 min). For all pressure levels used, DH followed similar trend in which an initial steep increase from 0 to 90 min was followed by a small drop off towards end of experiment (90-120 min). Increase in DH due to an increase in pressure holding time from 5 to 15 min (at 550 MPa) was found to be higher than increase in pressure level from 350 MPa to 550 MPa (5 min treatment) (p <0.05). For all pressure levels used, DH followed similar trend in which initial steep increase from 0 to 90 min followed by small drop off towards end of experiment (90-120 min). When high pressure was increased from 350 to 550 MPa, DH of EWP after enzyme hydrolysis increased from 6.73% to 7.84% (at 10% concentration) (Table 8.3) (overall 20% increase). On the other hand, when treatment time was increased at the maximum pressure level (550 MPa) from 5 to 15 min, DH increased from 7.84% to 11.41% (Table 8.2 and 8.3) (46% increase). Relative to samples without HP treatment, DH in samples treated with high pressure

showed a profound increase: 11 times the value of egg white protein without enzyme (11.41% vs. 0.93%) and 4 times for egg white protein with enzyme hydrolysis prior to HP treatment (11.41% vs. 2.25%). This clearly indicates advantage of using HPP for increasing the trypsin digestibility of food proteins. High-pressure treatment of egg white protein results in an increased digestibility of proteins with trypsin and therefore should increase the protein digestibility in the human system.

Table 8.3: **Effect of HP treatment on degree of hydrolysis of egg white proteins**

Time (min)	350MPa /5min	450MPa /5min	550 MPa /5min	550MPa /10min	550MPa /15min
0	2.86±0.007^{g}	3.20±0.08^{h}	3.42±0.02^{i}	4.73±0.03^{h}	5.11±0.008^{i}
15	3.39±0.022^{f}	3.77±0.05^{g}	4.16±0.02^{h}	5.49±0.021^{g}	6.01±0.004^{h}
30	3.95±0.04^{e}	4.39±0.02^{f}	4.78±0.04^{g}	6.18±0.05^{f}	7.02±0.002^{g}
45	4.87±0.007^{d}	5.34±0.015^{e}	5.74±0.02^{f}	7.13±0.03^{e}	8.06±0.004^{f}
60	5.88±0.02^{c}	5.91±0.038^{d}	6.50±0.03^{e}	8.4±0.002^{d}	9.08±0.01^{e}
75	6.76±0.04^{b}	6.99±0.04^{c}	7.35±0.02^{d}	9.151±0.015^{c}	10.24±0.02^{d}
90	7.10±0.04^{a}	7.54±0.015^{a}	8.25±0.03^{a}	10.14±0.016^{a}	11.34±0.03^{c}
105	6.74±0.018^{b}	7.36±0.015^{b}	8.04±0.02^{b}	10.17±0.02^{a}	11.59±0.03^{a}
120	6.73±0.03^{b}	7.39±0.05^{b}	7.84±0.01^{c}	9.97±0.009^{b}	11.41±0.03^{b}

Means within columns with same letters are not significantly different *($p>0.05$)*

The main reason for increased degree of hydrolysis could be attributed to unfolding of proteins chains (Zhu et al., 2006). HPP can cause dissociation and re-association of protein molecules by disrupting and further reformation of intermolecular bonds holding tertiary and secondary structure of proteins (Peñas et al., 2004). HP treatment was found to be responsible for dissociation of the soy protein into low molecular weight fraction; as a result the degree of hydrolysis was increased (Kajiyama et al., 1995). At moderately high pressures, it was recognized that digestibility of β-

lactoglobulin can be enhanced using trypsin enzyme, thermolysin and pepsin (Stapelfeldt et al., 1996). Correspondingly, protein solubility of kidney bean protein isolates was found to be significantly improved at pressure levels of 400 MPa or higher, due to arrangement of soluble aggregate from insoluble precipitates (Yin et al., 2008). The high pressure effects on soy and whey protein functionality was also demonstrated in our previous studies (Alvarez et al., 2008; Ahmed et al., 2007; Alvarez et al., 2007).

8.4.2 Effect of HPP followed by trypsin hydrolysis on antioxidant activity (AA) of EWP

Antioxidant activity of egg white proteins was evaluated using the DPPH assay method which is widely used to evaluate oxygen free radical scavenging effect of natural compounds (Yamaguchi et al., 1998). Effect of HP treatment on antioxidant activity of egg white protein before and after the enzyme hydrolysis was evaluated by the DPPH free radical scavenging activity method. In this method, DPPH radicals encounter the proton donating substances (antioxidant) which scavenge the free radical and results are shown as a reduction in the absorbance (Zhu et al., 2006). AA of proteins has been shown to increase as a result of change in their protein structure, concentration and reactivity. Table 8.4 shows Duncan grouping of mean value with standard error of AA for various egg protein samples. With increase in protein concentration from 2.5-10 mg/mL (2.5-10%), a significant (p <0.05) increase in AA was observed, i.e. from 5.64% to 9.34% (concentration increasing from 2.5 to 10%) without the trypsin hydrolysis and from 7.29% to 15.6% (concentration increasing from 2.5 to 10%) for egg white proteins after trypsin hydrolysis. P value shows that the increase observed was significant (Table 8.4). Enzyme hydrolysis by trypsin therefore was highly responsible for the enhancement of AA. Higher protein concentrations also contributed to an increase in AA. The intensification of antioxidant activities following the enzymatic hydrolysis of egg white proteins was in accordance with associated work showing increased antioxidant activity in egg yolk hydrolysates (Sakanaka et al., 2004). Hydrolysis of proteins will result in increase of antioxidant activity due to the reason that enzymes cleave peptide bonds at the interior of the polypeptide chain (Kabsch and Sander, 1983).

It was observed that antioxidant activity of proteins can be enhanced by increasing protein concentration but due to solubility issues, protein concentration cannot be increased above certain limit (10% w/v). On the other hand, enzyme hydrolysis of proteins can be used to amplify their antioxidant activity by increasing accessibility of amino acids with antioxidant potential to the pro-oxidants. Increase in antioxidant activity due to hydrolysis is mainly due to increased solvent exposure of amino acids (Kabsch et al., 1983).

Table 8.4: **Effect of enzyme hydrolysis on antioxidant activity of EWP**

Protein Concentration (%)	EWP without enzyme	EWP with enzyme
2.5	5.64 ± 0.028^{d}	7.29 ± 0.021^{d}
5	6.11 ± 0.07^{c}	8.30 ± 0.01^{c}
7.5	7.20 ± 0.05^{b}	11.34 ± 0.05^{b}
10	9.34 ± 0.02^{a}	15.64 ± 0.07^{a}

Means within columns with same letters are not significantly different *($p>0.05$)*

HPP has been recognized for increasing antioxidant activity of food components. Pressure induced changes can cause exposure of amino acid side chains and available peptide bonds (Messens et al., 1997). In this part of study, effect of HPP was evaluated on antioxidant activity of egg white protein. Table 8.5 explains the Duncan grouping of mean value and standard deviation of effect of HPP on antioxidant activity of egg white protein using different pressure treatments. The consequence of using increasing pressure levels (350-550 MPa/5min) on EWP resulted in increase in AA from 9.09% to 18.6%(350 MPa/5min) and 13.4% to 25.0% (550 MPa/5min) when the concentration was raised from 2.5 to 10% (p <0.05) (Table 8.5). AA showed increased from 18.6% to 30.7% (with increasing conc. 2.5-10%) for egg white protein treated at 550 MPa/15min followed by trypsin hydrolysis. Whereas in comparsion there was a smaller rise of 5.64%

to 9.34% and 7.29% to 15.6% in egg white protein without trypsin hydrolysis and with trypsin hydrolysis respectively at protein concentration of 2.5 -10 mg/mL (Tables 8.4 and 8.5). At maximum concentration of 10 mg/mL, AA was slightly enhanced from 18.6% to 25.0% with increase in pressure from 350 to 550 MPa for 5 min but when pressure treatment time was increased from 5 to 15 min at 550 MPa, there was almost 30% increase in AA i.e. from 25.0% to 30.7% (p <0.05). Increase in antioxidant activity of alkaline dephosphorylated phosvitin peptides has been observed (Volk, 2009). Enhanced hydrolysis /antioxidant activity of fish skin gelatin was observed when high pressure treatment was used in combination with alcalase or collagenase (Aleman et al., 2011).

Table 8.5: **Effect of HP treatments on antioxidant activity of egg white proteins**

Protein Concentration (%)	350MPa /5min	450MPa /5min	550Mpa /5min	550MPa /10min	550MPa /15min
2.5	9.09±0.01^{d}	11.90±0.02^{d}	13.38±0.02^{c}	15.46±0.01^{d}	18.58±0.03^{d}
5	12.40±0.05^{c}	16.42±0.02^{c}	17.66±0.04^{b}	21.07±0.002^{c}	22.48±0.03^{c}
7.5	17.42±0.05^{b}	19.09±0.02^{b}	21.42±0.02^{ab}	23.02±0.005^{b}	24.88±0.05^{b}
10	18.58±0.02^{a}	22.98±0.02^{a}	25±2.82^{a}	26.75±1.03^{a}	30.74±0.02^{a}

*Means within columns with similar letters are not significantly different *(p>0.05)*

It appears that HPP >200 MPa can cause rupture of non-covalent interactions in protein molecules which cause modification of tertiary and secondary structure of proteins (Messens et al., 1997). Antioxidant activity of proteins can be increased by disruption of tertiary structure (partial denaturation), thus increasing the accessibility of amino acids residue having antioxidant potential (Elias et al., 2008). Antioxidant activity of protein is dependent on amino acid composition. Many amino acids residues with antioxidant potential are buried in the protein core and their action is limited by the tertiary structure of polypeptides. High pressure can be used to increase exposure of these

buried amino acids by causing tertiary structure disruption. HPP can increase solvent exposure of amino acid thus increasing antioxidant activity (Elias et al., 2008)

Increased antioxidant activity of hydrolyzed food proteins has been reported for dairy (Ostdal et al., 2000) and egg yolk (Sakanaka et al., 2004). Hydrolysis is responsible for increased solubility of proteins in turn resulting in increased free radical scavenging activity. This modification by high pressure can enhance degree of hydrolysis and antioxidant activity of proteins. This increase in antioxidant activity by increasing degree of hydrolysis is the new functional characteristic of egg white protein hydrolysates. It can be used to supplement nutritional value of several foods and prevent deterioration of foods which are susceptible to oxidation.

8.4.3 SDS PAGE

SDS PAGE was used to evaluate the effect of high pressure on hydrolysis of egg white proteins. Results are shown in Figure 1, which shows the effect of trypsin enzyme hydrolysis (Part C) in untreated EWP and the same after HP treatment (Part D), on egg white protein. Egg white protein (Part B) was used as a positive control. It was observed that the positive control (egg white protein) shows two different bands corresponding to 34 kD (uncharacterized protein) and 14 kD (lyzozyme), which are absent in enzyme hydrolysed proteins before and after HP treatment. This confirms that the enzyme hydrolysis with and without HP treatment causes hydrolysis of protein structure; therefore no visible band was observed. This hydrolysis of protein causes breakdown of egg proteins into smaller peptides, which are not visible in SDS-PAGE. These results confirm that highest degree of hydrolysis occur in trypsin hydrolysed product after HP treatment of 550 MPa, causing production of small peptide bonds, is in agreement with highest degree of hydrolysis observed by OPA method. Similar observations were found in milk proteins for β-lactoglobulin proteins as they were hydrolysed using proteases and high pressure processing (Hayashi et al., 1987). Our results were backed up by another study on soy proteins where proteins were dissociated by using high pressure as confirmed by SDS-PAGE (Molina et al., 2002). These results supported by others (Galazka et al., 1996) deduced the conclusion that HP can effectively hydrolyse

egg white protein thus increasing degree of hydrolysis and in turn antioxidant activity. Thus, HP in combination with trypsin hydrolysis seems to be most viable tool to increase degree of hydrolysis and antioxidant activity.

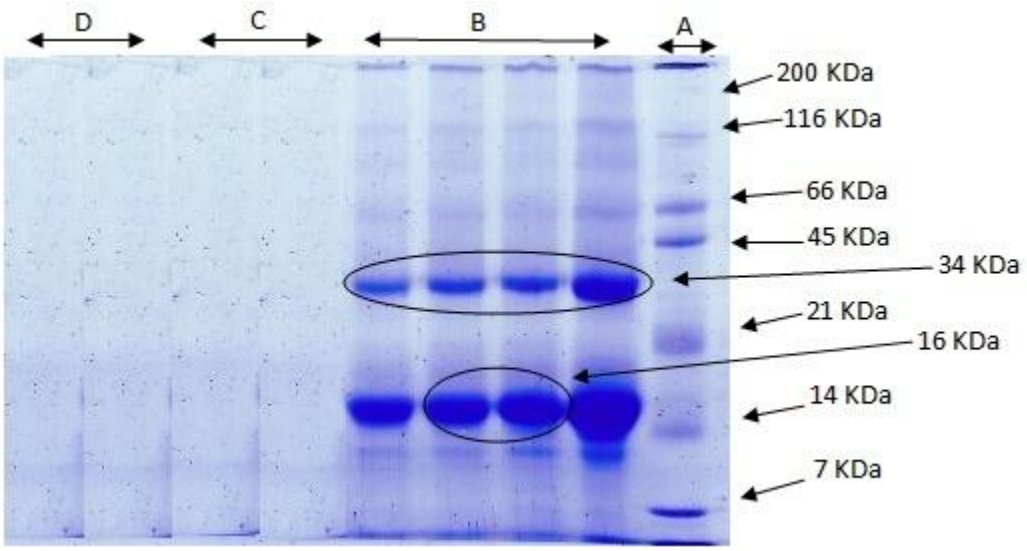

Figure 8.1: SDS- PAGE of egg white protein (EWP) (A= standard SDS reference, B=Isolated EWP (conc. 5mg/mL), C=EWP hydrolysate (conc. 5 mg/mL), D=HPP treated EWP hydrolysate)

8.5 Conclusions

Effect of high pressure processing and trypsin hydrolysis on degree of hydrolysis and antioxidant activity was studied. An increase in high pressure and holding time causes increase in degree of hydrolysis and antioxidant activity. Highest degree of hydrolysis was found at 550 MPa/15 min. By increasing holding time at higher pressure, the increase in degree of hydrolysis and antioxidant activity was more significant than with increasing high pressure treatment level. Protein breakdown was irreversible. EWH was showing higher degree of hydrolysis and antioxidant activity than EWP possibly due to breakage of bonds between protein structure and release of bioactive peptides which are responsible for the antioxidant activity.

CHAPTER 9

GENERAL CONCLUSIONS, CONTRIBUTIONS TO THE KNOWLEDGE AND FUTURE RECOMMENDATIONS

Overall, this study provides in-depth knowledge of rheological, structural and functional properties of various egg components. High pressure treatment causes modification of protein structure by denaturation which in turn affects the functionality. Impact of pressure varied according to composition of that particular egg component as the. egg yolk is high in cholesterol and egg white is rich in proteins. This study helps to better understand the changes occurring in egg components with increase in pressure treatment. There were number of changes that occurred in egg components in terms of structural and functional properties as they go from liquid to gel through semi-viscous through highly viscous and semi-solid phases. At higher pressure above 650 MPa, egg was even able to form self-holding and firm gels. It was observed that HPprocess-dependent parameters, like pressure level, treatment holding time and temperature exerted an important effect on determining HP induced gel network formation and extent of protein denaturation. HP-induced gelation at lower temperature can significantly improve the quality, taste and functional value.

The following are the specific claims of this research:

1. HP effects in combination with process variables like temperature and treatment time on flow properties were evaluated. HPP effects on denaturation of egg components were evaluated between liquid-viscous phase-solid gels by employing various methods of analysis including conventional rheology, back extrusion technique and texture profile analysis. This study was helpful in differentiating the pressure and time-temperature range which can be used to keep eggs in liquid, semi-viscous or gel stage depending on the final application.
2. HPP was used for formation of egg patties by inducing denaturation of egg white and whole liquid egg. High pressure was found to cause significant changes in various egg components and pressure level of 600 MPa or more was able to form fully formed gels. Textural properties (hardness, adhesiveness, cohesiveness,

springiness) were increasing with increase in treatment intensity for all egg components, but increase in EY was higher than other egg components. Pressure induced gels were soft, highly elastic and without any cooked flavor and taste.

3. The impact of process was also evaluated by inactivation kinetics of anti-nutritive enzyme present in the egg that can cause skin and thyroid related problems in humans. HP can cause inactivation of avidin at far lower temperature than thermal treatment (100°C) thus helping in preserving functional properties by preventing damage caused by heat.
4. HP was used to improve the functional value of egg white and whole liquid egg. Functional properties like water holding capacity and foaming behaviour were enhanced by pressure application.
5. Study of viscoelastic properties of egg components has provided detailed insight on elastic and viscous behavior. It also provided information about gel point behavior which ultimately translates to mouth feel.
6. It was found that HP can enhance the degree of hydrolysis of egg white proteins. Trypsin enzyme was used for hydrolysis of egg white protein and it was concluded that high pressure treatment causes partial unfolding of egg white proteins which helps in hydrolysis.
7. Antioxidant value of egg white proteins was increased due with HP application. HP-induced modification of egg white protein caused unfolding which exposes the suppressed peptides and amino acids responsible for antioxidant activity and it resulted in enhancement of antioxidant activity.

CONTRIBUTIONS TO THE KNOWLEDGE

1. Previous literature has only reported the thermal inactivation kinetics of avidin (pure avidin and avidin present in egg) ability to bind D-biotin. HP destruction kinetics of avidin was fully evaluated for the first time and compared with thermal inactivation. In this study, D and z- values were determined. It was found that HP treatments can cause faster inactivation of avidin than thermal treatment.

2. Few studies have been done on effect of HPP on rheology of liquid egg. Effect of HPP on egg rheology was fully characterized through all phase changes varying from liquid – semi solid – solide gel. Back extrusion rheology (new technique) was used for first time to bridge information gap between conventional flow rheology and texture profile analysis.

3. No work has been done before to evaluate influence of HPP on functional properties of egg white.This work has provided with the range of HP treatment conditions for various product formulations. HPP enhanced the water holding capacity and foaming characteristics of egg white.

4. Effect of HP treatment on trypsin hydrolysis of egg white protein was evaluated in detail demonstrating better digestibility of protein and expsoure of low molecular peptides chains which contributed to significant increase in their antioxidant activity.

Recommendations for future studies

1. Investigation of high pressure effects on egg components at molecular level for using lower temperature.

2. Improvement of viscoelastic properties by combining HP with low temperature. Investigation of impact of HP in combination with other enzymes such as chymotrypsin, trypsin and pepsin on degree of hydrolysis and antioxidant activity of egg proteins.

3**.** HP-induced egg gels can be used to entrap bioactive peptides and other important health benefiting compounds (vitamins) as HP doesn't cause any heat-induced damage to these components.

4. HP- induced egg patties can be used as a matrix to immobilize bacterial spores.

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