FACTORS INFLUENCING THE FORMATION OF ZEIN AND GUM ARABIC COMPLEX COACERVATES

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Complex coacervates are mixtures of biopolymers such as proteins and polysaccharides. The objectives of this research were to (1) determine the optimum biopolymer ratio and pH for the formation of Zein protein and gum arabic complex coacervates, (2) determine the stability of Zein: GA coacervates as an emulsifier using flax oil, transglutaminase (Tgase), Tween 80, and Span 80 (surfactants), and (3) determine optimum temperature for the stability of formed Zein: GA complex coacervates. The optimum ratio, pH and temperature were determined using turbidimetric and zeta (ζ) potential analysis. Analysis confirmed the formation of stable Zein: GA coacervates at ratio 2:1, at pH 4.5 ± 0.05 and most stable at temperature 25 ± 2 °C. Zeta (ζ) potential analysis also confirmed the formation of stable emulsion using Zein: GA coacervates at 5% Tgase and 25% Span 80. Therefore, Zein: GA complex coacervates could be used as an emulsifier in food industry.

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DEDICATION

To my beloved parents, brother and sister

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1. INTRODUCTION

Biopolymers (protein and polysaccharides) are an essential part of biological systems, as they provide essential energy and metabolite for the basic activities of living systems. Each biopolymer has its respective function based on molecular structure. One of the structures formed by biopolymers mixture is known as complex coacervation. According to Kizilay and others (2011), coacervation leads to the separation of two immiscible liquid phases since solution is composed of two oppositely charged macroions (colloids). These coacervates are stabilized by an interaction between them due to opposite surface charge known as electrostatic interaction (Liu and others 2010). Different factors, such as pH, temperature, and salt concentrations affect the surface charges on these biopolymers and hence coacervate stabilities. A surface charge on a particular biopolymer attracts other biopolymers with opposite charges. This attraction is also called thermodynamic compatibility. Properties exhibited by formed coacervates depend on biopolymer characteristics (e.g., type, molecular weight), concentration, and solvent conditions (e.g., pH and temperature) (Schmitt and Turgeon 2011).

One of the applications of these formed coacervates is microencapsulation, a process by which solids, liquids or even gases may be enclosed in microscopic particles like coacervates. Particles based on these coacervates are used in the food industry to protect vitamins or flavors for food delivery (Agnihotri and others 2012). They are made up of food-grade ingredients (proteins and polysaccharides), and the products are biodegradable and non-toxic (Matalanis and others 2011).

The most widely used complex coacervate for encapsulation is made up of gelatin and gum arabic, for microencapsulation purposes. Recently, Zein protein from corn (*Zea mays* L.) was used to form simple coacervates and was further used for encapsulation of a drug called gitoxin (Muthuselvi and Dhathathreyan 2006). In food industries, Zein has been widely used for its film forming properties, but the limitation with this protein is its poor emulsification properties (Shukla and Cheryan 2001). Therefore, this research aimed to determine emulsifying properties of Zein protein by mixing gum arabic to form complex coacervates.

Coacervates and their formation have significant importance for food and drug industries. The objectives following the study are to determine: (1) the optimum biopolymer ratio and pH for the formation and stability of Zein protein and gum arabic complex coacervates, (2) the stability of mixture of flax-oil, water and Zein: GA coacervates with transglutaminase (Tgase), Tween 80 and Span 80 (surfactants) concentrations to stabilize emulsions, and (3) the impact of temperature on the stability of formed Zein: GA complex coacervates.

2. LITERATURE REVIEW

2.1. Importance and function of prolamin protein in cereal seed

2.1.1. Cereal storage protein

Seed proteins are usually classified into three different groups based on their biological role: storage proteins, structural and metabolic proteins, and protective proteins. The storage proteins act as discrete bodies where macronutrients such as nitrogen could be stored. These macronutrients play an essential role in growth of plants and maintain storage proteins or others synthesis in plants (Shewry and others 1995). Classification of different proteins present in cereal was conducted using sequential extraction based on their solubility into four components: albumins (soluble in water), globulins (soluble in dilute salt solutions), prolamins (soluble in aqueous alcohol) and glutelins (soluble in dilute acids or alkalis) (Osborne 1907; Koehler and Weiser 2013).

Albumins and globulins are widely distributed among flowering plants, i.e. seed producing plants, these proteins are considered as metabolic proteins, as their basic function is to provide nutrition to embryo during germination. Prolamins and glutelins are classified under class of storage proteins; their basic function is to provide nitrogen for plant growth. Prolamin presence is limited to grass family i.e. wheat (*Triticum aestivum* L.), maize, barley (*Hordeum vulgare* L.) (Koehler and Weiser 2013). In all cereals, except for rice (*Oryza sativa* L.) and oats (*Avena sativa* L.), prolamins make up the major endosperm storage proteins. In oats and rice, globulins form major endosperm storage protein fraction, for about 70-80% of total protein (Shewry and Halford 2002).

Osborne, 1907 named the major storage proteins in cereal as prolamin; it reflects the presence of high contents of proline and glutamine (i.e. pro for proline and –amin for glutamine). In cereals, storage proteins were based upon their Latin generic names. For example, prolamin in maize (Zea mays L.) was named as Zein (Shewry and Tatham 1990). Prolamin present in wheat is named as gliadin, in oats is avenin and in barley it is called hordein (Delcour and Hoseney 2010).

Cereals proteins are extensively used for encapsulation purposes, as these naturally occurring polymers: these biopolymers are biocompatible and biodegradable. Currently, animal extracted proteins are widely used as microparticles for encapsulation, but cereal proteins are more advantageous. Addition of cereal proteins would be nutritious and less allergenic to consumers compared to animal extracted proteins. Adults and infants may develop allergic to seafood animal proteins and infants to cow's milk, respectively (Nesterenko and others 2013). Prolamin proteins from cereal have always been used for encapsulation purposes due their storing capacities. Recently, Zhang and others (2014) prepared thymol-loaded Zein nanoparticles stabilized by using sodium caseinate (SC) and chitosan hydrochloride (CHC) double layer. The study concluded that nanoparticles exhibited stronger antimicrobial activity and also presence of prolamin as microparticles could be beneficial for food industry for encapsulation.

2.1.2. Structure and properties of prolamin

The basic anatomy of most cereal kernels is similar, for example maize kernel is divided into different parts: endosperm, germ, tip cap and pericarp. The endosperm consists of starchy endosperm and aleurone layer (Belitz and others 2009). In starchy endosperm, prolamins are deposited as discrete protein bodies as they account for about half protein present in the mature

grain (Shewry and Tatham 1990). Originally, prolamin's name was based on its rich proline and glutamine amino profiles. Proline is incapable in forming hydrogen bond with other amino acids due to presence of nitrogen in its structure, resulting in formation of prolyl peptide bond with limited rotation as it forms the backbone of protein structure; thus presence of proline residues reduces the structural flexibility (Cox and Nelson 2008; Shewry and Tatham 1990). The presence of high percentage of proline amino acid in the prolamin's composition leads to the hydrophobic nature of prolamin as it limits formation of hydrogen bond formation with water molecules and also responsible for its storage capabilities (Simpson 2001). prolamins makes it a distinct group of cereals storage proteins i.e. its tendency to be soluble in alcohol-water mixtures and insoluble in water except at high concentrations of urea (Shukla and Cheryan 2001).

According to Delcour and Hoseney (2010), in wheat one of the group of prolamins present are called gliadins, their composition is rich in glutamine and proline and also high percentage of leucine, valine, serine, isoleucine and phenyalanine is present. The structure of α, β, ɣ-gliadins is stabilized by interchain disulfide bonds, whereas ω-gliadins are incapable of comprising disulfide contain due to the absence of cysteine residues. The other group is classified as glutelins, consist of disulfide bonded polymers and are further consists two groups i.e. high molecular weight (HMW) and low molecular weight (LMW) subunits (Eliasson and Tatham 2001).

2.2. Structure and properties of maize protein (Zein)

2.2.1. Classification maize protein (Zein)

In maize (*Zea mays* L.), protein occurs in endosperm and mainly composed of a prolamin called Zein. Maize's endosperm contains about 44% Zein and 28% of glutelins, whereas

albumin and globulins are about 5%. Zein present in maize does exhibits similar properties and structure as it's belongs to family of prolamin. For example; Zein is alcohol-soluble protein, basic structure of this protein is rich in proline and glutamine. This protein also is low in essential amino acids such as lysine and tryptophan (Delcour and Hoseney 2010).

Different advancement was made regarding Zein's structure and all indicated that Zein is a mixture of different peptides; these peptides were differentiated based upon their molecular size, solubility and charge. McKinney (1958) described the first two major peptides of Zein, i.e. α- and β- Zein. He described α- Zein as 95% ethanol soluble maize prolamin and this fraction represents about 80% of total prolamin in maize. Paulis and others (1969) showed that α-Zein represents about 35% of total protein and had two major bands of 24,000 and 22,000 MW. Then, Pomes (1971) analyzed maize using starch gel electrophoresis, he described that β-Zein could have higher molecular weight than α- Zein due to the formation of disulfide linkages between α-Zein molecules. As after using reducing agent, β-Zein migrated in the starch gel and revealed three different bands of 24,000, 22,000 and 14,000 Da.

Esen (1986, 1987) proposed nomenclature of Zein's various fractions based upon their solubility. It was proposed that Zein peptides could be fractioned based upon their solubility, amino acid structure in solutions containing 0 and 95% isopropyl alcohol (IPA), adding reducing agents and buffers. First three fractions obtained were named as α, β and ɣ- Zein. Initial fraction i.e. α- Zein, it was soluble in 50-95% IPA, but insoluble in 30% IPA/30mM Na- acetate.α-Zein contains high percentage of hydrophobic residues such as proline, alanine, leucine and phenylalanine (Gianazza and others 1977).

According to Esen (1987) and Larkins (1989), it accounted for about 75-80% of total Zein in maize, but percentage also depended upon the genotype. Whereas next fraction was named as β-Zein, soluble in 30-95% IPA that contained a reducing agent, but insoluble in both 90% IPA and 30% IPA/30mM Na-acetate. This fraction accounts for 10-15% of total Zein and is made up of two17, 000-18,000 MW methionine-rich polypeptides. Last was fraction was named as ɣ-Zein, which is soluble in 0-80% IPA (0 with no reducing agent) in presence of reducing agent and it was also soluble in 30% IPA/30mM Na-acetate. This fraction accounts for 5-10% of total Zein and is made up of one 27 000 MW proline-rich polypeptide.

Another fraction which was later named was δ-Zein. This fraction small portion of total protein, it is also rich in sulfur amino acids, methionine and cysteine (Wallace and others 1989; Kirihara and others 1990). Then it was proposed that these polypeptides represent the primary source of sulfur storage in the maize seed (Pedersen and others 1986). Savich (1991) proved that Zein's hydrophobic nature was due to presence of larger peptides. As larger the molecular weight of peptides higher will be hydrophobicity as due to presence of high number of non-polar amino acids.

According to Lending and Larkins (1989), maize's endosperm's interior region contains α-Zein whereas outer cell layers have a higher concentration of β- and y-Zein. So, later it was proved that, α- and δ- Zein are mostly found in the core region, whereas β- and ɣ- Zein are on the periphery region of the maize protein body. Osborne (1924) considered β- and ɣ- Zein fractions as glutelins whereas α- and ɣ- as true prolamin.

2.2.2. Structure of Zein

As explained above, Zein is classified into four peptides- α, β, ɣ and δ- Zein, based upon their solubility. These peptides also differ in their amino acid sequence. α- Zein is most abundant peptide in protein body for about 80% and contains two major bands of molecular weight 24,000 and 22,000 Da (Sharma and others 2012). Kretscmer (1957) explained Zein using infrared spectroscopy and proposed that Zein's content of α-helical is about 50 % in 80 % of ethanol, and also suggested the presence of pleated sheets (β- sheets). Danzer and others (1975) used optical rotatory dispersion measurements to describe the structure of Zein protein in non- aqueous solvent. Based upon the helical content it was concluded that Zein is a globular protein in non-aqueous solutions. But, Zein in comparison with other conventional globular protein such as insulin and ribonuclease has variation in secondary structure though is similar in conformational properties.

Argos and others (1982) investigated molecular conformation of α- Zein (Z19 and Z20) using circular dichroic data. It was proposed that Zein's α- helical structure of protein and the presence of 50-60% of α-helical content and also configuration of random coil was confirmed. Z19 and Z20 displayed homologous amino acid sequence; in both structures 20- amino acid residues were repeated nine times, as they were arranged in an anti-parallel form forming α-helix and having three non-polar segments on its surface. The turns in random coil structure were also investigated and concluded to be rich in glutamine. Each turn was stabilized by hydrogen bonds and Van der waal interactions among neighbor α- helices using polar (glutamine) segments. Arrangement of Zein molecules like rod-shaped explains its property of behaving as a storage protein, as Zein molecules aggregates in molecular planes; they could stack through interaction formed between glutamine present at cylindrical caps.

Later Matsushima and others (1993) modified Argos and others (1982) α-helical structure by using small-angle X-ray scattering measurements (SAXS). Further investigation proved that the α- helical segments of Zein are held by hydrogen bond and arranged in prism-like shape instead of a rod-like shaped. Momany and others (2006) preferred the rod-shaped α-helical to further characterize the N-terminal of Z19. The results predicted the presence of cysteine residue on the surface of Z19 and available for the formation of disulfide bridge with cysteine residue present on surface of Z22 protein.

Recently, Zhang and others (2011) investigated the effect of acid and base on structural and antioxidant properties of α- Zein. The results collected from infrared spectroscopy indicated decreased contents for α-helix, β-sheet, and β-turn under proper acidic or basic conditions, which lead towards changes in structural and antioxidant properties, and further contributed to deamination of glutamine to glutamic acid. This results in higher antioxidant properties but decreased viscosity of Zein. Zein's tertiary structures allow it to self-assemble into chains and layers. These properties have been exploited to form aggregates and entrap solutes (Sousa and others 2012), and Zein is thus used commercially as an edible coating and protective layering in drug and food delivery systems (Muthuselvi and Dhathathreyan 2006).

2.2.3. Uses of Zein

Zein protein as contains high amount of proline amino acid, due to this protein-based films are less susceptible to microbial attack, which makes Zein protein a useful tool for food industry. Zein can form greaseproof films that are tough and resistant to microbial attack films, and is used as a biopolymer for coatings. Zein has been used for various purposes such as production of adhesives, biodegradable plastics, chewing gums, cosmetic powders, but this

protein was also widely used for microencapsulation using different techniques (Shukla and Cheryan 2001).

Initially, method to encapsulate drug by forming protein microspheres was introduced by Mathiowits and others (1991). Drugs were included into protein microspheres to deliver it to gastrointestinal tract. Many research were conducted regarding Zein as an encapsulation tool, out of them few are discussed. Heparin- a drug used to treat cardiovascular diseases was used in Zein-based microspheres, and these drug loaded microspheres proved to be effective in platelet adhesion and displayed anticoagulation (Wang and others 2005). Muthuslevi and Dhathathreyan (2006) used Zein for simple coacervates formation to encapsulate gitoxin drug, and it was concluded that Zein-based microspheres were suitable for sustained-release of gitoxin drug. Later, Zhang and others (2009) concluded that Zein-fish oil displayed better oxidative stability in 90% isopropanol and a good alternative for emulsion. A recent article published by, Moomand and Lim, regarding encapsulation of fish oil using Zein fibers using an electrospun microencapsulation technique. They concluded that encapsulation efficiency reached up to 91% for ethanol-based and 96% for isopropanol-based Zein fibers (Moomand and Lim 2014).

2.3. Introduction to microencapsulation

Microencapsulation is a process by which solids, liquids or even gases may be enclosed in microscopic particle. The process has begun in the late 1930s as a cleaner substitute for carbon paper and carbon ribbons as sought by the business machines industry. The ultimate development in the 1950s of reproduction paper and ribbons that contained dyes in tiny gelatin capsules released on impact by a typewriter key or the pressure of a pen or pencil was the stimulus for the development of a host of microencapsulated materials, including drugs.

A well designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug. To obtain maximum therapeutic efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount in the right period of time there by causing little toxicity and minimal side effects. There are various approaches in delivering a therapeutic substance to the target site in a sustained controlled release fashion. One such approach is using microspheres as carriers for drugs.

Microspheres are characteristically free flowing powders consisting of proteins or synthetic polymers that are biodegradable in nature and ideally having particle size between 200 - 800µm (Agnihotri and others 2012). The microencapsulation technique widely being used for drug or flavor delivery is complex coacervation or also known as thermodynamic compatibility.

2.3.1. Formation of protein-polysaccharide complex coacervates

Coacervation is defined as a process during which a homogenous aqueous solution of charged macromolecules, undergoes liquid-liquid phase separation, giving rise to a polyelectrolyte-rich dense phase (Bohidar 2008). This technique was the first encapsulation process studied and was initially employed by Green and Scheicher (1955) to produce pressure-sensitive dye microcapsules for the manufacturing of carbonless copying paper. Coacervates, were first named by Bungenberg de Jong (1949), are formed when mixed dilute solution of gelatin and an anionic polysaccharide (acacia, pectin) is brought to a pH at which polyelectrolyte have opposite net charges (Bungenberg de Jong 1949).

The electrostatic interaction between oppositely charged polysaccharides may result in coacervation, if at least one of macromolecules is not as strong polyelectrolyte or internal charges are not accessible. This interaction falls into the classification of complex coacervation,

which involves more than one colloidal solute (Coelho and others 2011). When mixed, proteins and polysaccharides form complex coacervates or associative phases (thermodynamic compatibility) in a narrow pH range. Since electrostatic interactions is main driving force between protein and polysaccharide and this lead towards associative phase separation or coacervation. Other entropic factors (e.g. molecular charge density) also influences phase separation or formation of coacervates.

Complex coacervation is driven by entropy gain that arises from molecular rearrangements that occur during electrostatic interaction in order to form a random aggregated phase. Intrapolymeric complexes still carry a negative charge, and may remain charged until pH of the solution is below protein's isoelectric point, this change in pH i.e. charged ions allow them to remain soluble into the solvent. Therefore, complex coacervates involve spontaneous separation into coexisting solvent- rich and solvent depleted phase, the latter consists of a co-precipitate of both biopolymers (Dickinson 1995). The above explained interaction leads towards the formation of thermodynamic compatibility strucutres. On the other side, when both molecules carry similar charges this could result in electrostatic repulsion and further into the formation of two different phases, one rich in protein and one rich in polysaccharide.

Biopolymers are oppositely charged in solution, which results in two phases: a solvent phase and a biopolymer rich phase or droplets of coacervate. The solubility of proteins within mixtures of proteins and polysaccharides under associative phase separation depends on biopolymer-biopolymer and biopolymer-solvent interactions; solubility depends on the overall surface charge of the formed complexes, which in turn is related to surface hydrophobicity, the biopolymer ratio, and solvent conditions (Schmitt and others 1998). Basically, formation of coacervates improves individual biopolymer functionality by synergistic interactions between

protein and polysaccharide, with repercussions for the stability, texture, and shelf-life of many food products.

Most of the food dispersions are in form of foams or emulsions, due to the interactions between protein-polysaccharide in the aqueous phase, it leads to effect adsorption on protein interfacial (Pérez and others 2009). Stabilization or destabilization of oil in water emulsions in protein-polysaccharide mixtures depends on the nature of the biopolymers, solvent conditions, and biopolymer concentrations (Vikelouda and Kiosseoglou 2004). As levels of polysaccharide increase in mixed biopolymer systems, formation of a network-like structure within the continuous phase enhances emulsion stability of overall structure (Papalamprou and others 2005); at higher polysaccharide concentrations, 'steric stabilization' is favored as multiple polysaccharides complex to and saturates protein stabilized interfaces (Dickinson 1998).

Formation of these compatible structures does depend on different factors, such as pH of solution, mixing ratio, biopolymer concentration, temperature and biopolymer characteristic (type, molecular weight).

2.3.1.1. Effects of pH on complex coacervation

The pH is one of the factors that influence the formation of coacervates. Rise or decline in pH of the solution leads towards modifications in surface charge upon protein and polysaccharides and leads towards the initiation of coacervates formation. Change in pH directly influences the gain or loss of negative or positive charges of functional side groups present on both biopolymers (i.e. amino and carboxylic groups) (Schmitt and others 1998, 2009; Ye 2008). As pH approaches below the isoelectric point (pI) of the protein, the net opposite charges between two biopolymers increases which leads towards the formation of electrostatic force

stabilized complex (Leward 1979). Usually, cereal proteins such as Zein has pI ~ 6.8 and anionic polysaccharides for example gum arabic has pKa ~ 2.2, interacts with each other within intermediate pH range i.e. from 3.0-5.0 (pKa<pH<pI) (Dickinson 1998; Ye 2008).

Liu and others (2009) described the complex coacervation as a pH induced structure forming event that includes the formation of soluble or insoluble complexes (denoted as pHc) and this structure-forming event is generally accompanied by a commencing increase in turbidity. Further decreases in pH leads towards the formation of insoluble complexes and is accompanied by larger changes in turbidity (denoted as pHø1). Further decrease in pH leads maximum formation of coacervate accompanied by maximum turbidity (denoted as pHopt). Since pH of the solution comes closer to polysaccharide's pKa value leads to dissolution of complexes since acidic pH increases the positive charges on amino groups present on the backbone of the polysaccharide (denoted as pHø2). The structure of polysaccharide and the pH induce changes in the protein conformation, results in distinctive surface properties of the biopolymer complex formed.

For this study, acidification process was conducted using glucono-δ-lactic acid (GDL). Usually such acidification method is commonly used by dairy industry (Braga and others 2006). As direct addition of lactone i.e. using GDL leads to stable formation of casein (milk protein) colloids formation in milk (Braga and others 2006). GDL (glucono-δ-lactic acid) is also called D-gluconic-δ-lactone is an internal ester (cyclic 1, 5 intermolecular ester of D-gluconic acid). Upon addition to milk, it hydrolyzes and forms gluconic acid and lactone rings, but gluconic acid further dissociates into hydrogen ions (H+), therefore decreases pH of the solution (Thomas and others 2008; De Kruif 1997). In this study, GDL was used to stabilize formation of Zein: GA complex coacervates, as GDL results in slower rate of acidification relatively to other acidulates

(Thomas and others 2008). Besides pH of the solution, there are other factors which also contribute towards the formation of complex coacervates, which includes mixing ratio of both the biopolymers and temperature of the solution.

2.3.1.2. Effect of mixing ratio

Weinbreck and others (2004) reported that biopolymer mixing ratio (r) had a major effect on the characteristics of behavior of formed coacervates because of its dependence upon charge balance of formed coacervates. Schmitt and others (2001) also determined the optimum mixing ratio of β-lactoglobulin/acacia gum coacervates using diffusion wave spectroscopy (DWS). Protein: polysaccharide (2:1) exhibited both coalescence and sedimentation whereas at ratio 1:1 stable particles were formed. Another study was conducted by Sanchez and Renard (2002) to investigate the stability and structure of β-lactoglobulin/ acacia gum coacervate in the presence of protein aggregates at pH 4.2 and at ratio 8:1, 2:1 and 1:1 Protein: polysaccharides. They determined that formed protein aggregates were interacting with complex coacervates to keep them stabilized.

Recently, Yuan and others (2014) investigated the influence of pH, mixing ratio, heat treatment and ionic strength on coacervates formed using soy protein fractions and chitosan. For this study, zeta-potential was used, to understand the stability of the mixture. They concluded that respective parameter was least affected by heat treatment and mixing ratio compared to ionic strength; as changes the in first two factors only affected the equilibrium between forces forming coacervates.

Effect of mixing ratio was also explained by Turgeon and Laneuville (2009). According to them if one of the biopolymers is in excess compared to another; it could lead to insufficient

charge neutralization i.e. formed coacervates could remain charged over wide range of pH. Mixing ratio also has critical influence on the structure of formed coacervates. A low mixing ratio, small coacervates are formed which rapidly coalesce into large coacervates. At high mixing ratio, coalesce did not occurr rapidly (Schmitt and others 2009; Turgeon and Laneuville 2009).

2.3.1.3. Effect of temperature

Further the effect of temperature on formation of coacervates is discussed. As temperature is also one of factors which effects formation and stability of coacervates (Ye 2008). Processing factors such as temperature affects the formation and stability of the formed coacervates (Ye 2004). Increase/decrease in temperature can cause conformational changes in protein or polysaccharides and also alter interactions between protein/ polysaccharide and solvent (Mizusaki and Morishima 1998). Ye (2008) also described that due to temperature increase, hydrophobic interactions also enhances with covalent bonding, whereas decrease in temperature is more favorable towards hydrogen bonding. But increase in temperature, is usually causes denaturation of globular proteins and conformational changes in polysaccharides. These changes leads towards the exposure of reactive groups present on biopolymers and hence favor complex interactions.

2.4. Structure and properties of gum arabic

Gum arabic (GA) or Acacia gum is found in nature as a tree exudate gum, i.e. it is extracted from tree widely grown in Africa known as *Acacia senegal* or from its species. Usually, gum arabic is secreted under stress conditions such as drought conditions in the particular area from stem and branches of tree (William and Philips 2000). Anderson and

Stoddart (1966) described the structural features of gum arabic, basically it is an acidic heterogeneous polysaccharide, composed of monosaccharides such as galactose, rhamnose, and arabinose and also glucuronic acid, all branched together. Idris and others (1998) concluded that gum arabic from Acacia senegal contains ~ 42% of galactose, ~ 25% arabinose, ~ 15% rhamnose and 15% of glucuronic acid. But it was also established that ~ 2% protein, but gum extracted from another species of *Acacia* i.e. *Acacia seyal* contains lower proportion of nitrogen compared to senegal (William and Phillips 2000; Idris and others 1998).

2.4.1. Introduction of gum arabic (GA) structure

The structure of gum arabic was initially described by Anderson and Stoddart (1966), though later number articles were published based upon its structure, but two of the recent review articles published were by Verbeken and others (2003) and then Ali and others (2009). According to articles referred before, gum arabic is composed of 1, 3-linked β-D-galactopyranosyl units. Both the main and the side chains in structure are joined together and contain units of α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D- glucuronopyranosyl and 4-O-methyl- β- D- glucuronopyranosyl. Primarily consists of two major fractions constituting one which contains ~ 80% of polysaccharide chains i.e. monosaccharaides branched together and another fractions ~ 10% of polysaccharide contains molecules of higher molecular weight that contain ~ 2% protein i.e. complex of arabinogalactan and protein (Montenegro and others 2012).

2.4.2. GA as an emulsifying agent

Gum arabic (GA) has been used widely in food industry especially for beverages, because of its emulsifying properties. Randall and other (1988) concluded that the fraction that predominantly absorbed at the oil-water interface was heavy molecular weight indicating that

protein fraction plays a major role in emulsifying property of entire gum arabic structure as it contains hydrophobic group whereas hydrophilic groups are present on monosaccharide fraction oriented towards aqueous phase (Randall and others 1988). Later McNamee and others (1998) investigated emulsion and microencapsulation properties of gum arabic using spray-dry technique. They concluded that average particle size of formed spray-dried emulsions was within the range 9-17 μm, based on oil/gum ratio, microencapsulation was observed till 100%, and also final powder product up to 50% oil content were readily dispersed in water.

Verbeken and others (2003) indicated that the heterogeneous structure of gum arabic makes it an excellent emulsifier. For this study, gum arabic will best match for coacervate formation because it will enhance properties of Zein when mixed with it. As Zein can form thick films but cannot act as emulsifying agent whereas gum arabic will provide such properties to formed coacervates.

3. MATERIALS AND METHODS

3.1. Study 1 (Formation of Zein: GA coacervates: effect of pH and biopolymer ratio on their formation and their stability)

3.1.1. Materials

Zein (Maize, Lot#SLBB1867V) and gum arabic (GA) (Acacia tree, Lot#11229) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The composition of Zein was ~ 12% moisture, 80.16% protein (%N×6.25) and 9.3% fat. In contrast, gum arabic was composed of 1.84% protein (%N×6.25); 3.02% fat and ~ 8% moisture. Chemical analysis on all materials were conducted according to AOAC methods 925.10 (moisture), 920.87 (crude protein), and 920.39C (Cereal fat). Transglutaminase (from guinea pig; Lot#SLBF9464V2UN, ≥ 1.5 units/mg protein), Tween 80 (Lot#MKBP2328V) and Span 80 (Lot#BCBD2426V) was also purchased for Study 2. Flaxseed oil used to perform emulsion test was obtained from a local food store in Fargo, ND. All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). Water was purified by a Milli-Q water system with 18 MΩ or greater (Millipore, Milford, MA) and used for all experiments. All ingredients were used without further purification.

3.1.2. Formation and stability of Zein: GA complex coacervates

The procedure for complex coacervate formation was adapted from Juttulapa and Sriamornsak (2012) and Liu and others (2010).

3.1.2.1. Preparation of stock solutions

Biopolymer stock solutions (1% w/w; pH 7.0) were prepared by separately dispersing gum arabic and Zein powders in Milli-Q water and 70% (v/v) ethanol (EtOH) respectively, with

each stirred individually at 400 rpm for more than 6 hr to ensure complete hydration. Zein and gum arabic solutions were cooled to 4 °C overnight to help facilitate protein solubility. The Zein solution was then centrifuged at 7,500 rpm for 20 min and the GA solution centrifuged at 8,500 rpm for 30 min to remove insoluble particles.

3.1.2.2. Turbidimetric analysis by using acidification of Zein: GA complex coacervates

Mixtures of Zein and GA were prepared by mixing appropriate masses of stock solutions, at the desired ratios of Zein: GA (1:1, 1:2 and 2:1), Milli-Q water was used to maintain total biopolymer concentration of 5% (v/v) (denoted as C). Initially turbidity measurements were performed using different Zein: GA ratios at 1:1, 1:2 and 2:1 over a pH range of 7.0 ± 0.05-2.5 ± 0.5 using a UV/VIS spectrophotometer (Shimadzu Co, Torrance, CA) at 310 nm. The pH values of the mixtures were adjusted using 0.5% (w/v) gluconic- δ-lactone and 0.1 M NaOH. Milli-Q water was used as blanks. All measurements were performed in triplicates.

3.1.2.3. Zeta (ζ) potential (mV) of Zein: GA complex coacervates

Zeta (ζ) potential of Zein: GA coacervates were carried out with a dynamic light scattering type ZetasizerNano -ZS90 (Malvern instruments, U.K.) apparatus equipped for protein size measurement sensitivity. Samples for zeta analysis were prepared in similar as prepared for turbidimetric analysis (5% v/v total biopolymer concentration at 2:1 ratio) at different pH values from 7.0 ± 0.05-2.5 ± 0.5. Vertical cuvettes with a path of 10 mm were used as scattering cell. Measurements were performed at a scattering angle of 90 ° from two different directions, at room temperature (25 ± 1 °C). Measurements for each sample were taken in triplicates. Each replicate was obtained from 20 measurements cycles.

3.1.3. Characterization of formed Zein: GA coacervates using microscope

Samples for photomicrographs were prepared by mixing Zein and gum arabic at ratio 2:1 and deionized water was used to maintain total biopolymer 15% (v/v) at pH 4.5 ± 0.05 by slow addition of 0.5% (w/v) glucono-δ-lactone. Two separate samples were prepared using Zein 15% (v/v) and gum arabic 15% (v/v) samples at pH 4.5 ± 0.05 as controls. Differential Interference Contrast (DIC) photomicrographs were obtained using 100x/1.45 alpha Plan-Fluar objective on Zeiss Axio Observer Z1 inverted scope equipped with Zeiss Axio Cam MRc Rev3 camera and Zeiss Axio Vision Rev. 4.8.1 microscope control and image analysis software (Carl Zeiss, Thornwood, NY)

3.1.4. Statistical analysis

Experimental design for both readings obtained from spectrophotometer and zeta sizer, sample layout was absorbance and zeta as dependent variables in completely randomized design with no sampling with three replicates. Initial set of readings were obtained involving two factors, pH and ratio and with three replicates; whereas for zeta readings analysis was single-factor experiment in completely randomized design with three replicates and each replicate contained 20 measurement cycles. Analysis of variance was performed using ANOVA procedure in SAS for Windows (Version 9.3, SAS Institute Inc.,Cary, NC).

3.2. Study 2 (Determination of the stability of emulsion of flax-oil formed using Zein: GA complex coacervates using transglutaminase (Tgase), Tween 80, and Span 80)

3.2.1. Materials

Same materials were used for this study as described in 3.1.1.

3.2.2. Preparation and characterization of oil-in-water emulsion

The procedure of preparation of oil-in water emulsion was adapted from Lvand others (2014) and McClements (2009).

3.2.2.1. Transglutaminse (Tgase) activity for stabilization of Zein: GA coacervates

Tranglutaminase activity was determined by using initial concentration at 0.25% (w/v). For this experiment, different percentages of Tgase were used (0, 1, 5, 10, 25, 50, 75) of total biopolymer concentration (0 is control). Deionized water was used to maintain total biopolymer concentration i.e. 5% (v/v). Initially, Zein: GA coacervates were prepared at ratio 2:1 and at pH 4.5 ± 0.05, by slow addition of 0.5% (w/v) glucono-δ-lactone and kept at room temperature (25 °C) for 1 hr. After addition of Tgase enzyme, solutions were kept on orbit shaker (Model 3520, 120V; Lab-Line Instrument Inc., USA) at 400 rpm for 3 hr at room temperature (25 °C). For stability of Zein: Ga coacervates Zeta (ζ) potential analysis was carried. Using same procedure followed in 3.1.2.3.

3.2.2.2. Percentage of Tween 80 and Span 80 for stabilization of Zein: GA coacervates

For this experiment, different percentages (0, 1, 5, 10, 25, 50, 75, 100) of Tween 80 and Span 80 at different ratios (1:0, 1:3, 1:1, 3:1, 0:1) were used of total biopolymer concentration (0 is control). Initially, Zein: GA coacervates were prepared at ratio 2:1 and at pH 4.5 ± 0.05 by slow addition of 0.5% (w/v) glucono-δ-lactone, using 5% Tgase of total biopolymer concentration. Deionized water was used to maintain total biopolymer concentration i.e. 5% (v/v). For stability of Zein: Ga coacervates zeta (ζ) potential was carried using same procedure followed in 3.1.2.3.

3.2.2.3. Preparation of oil-water emulsion

In this experiment, different percentages (0, 1, 5, 10, 25, 50, 75, 100) of flax oil of total biopolymer concentration were used (0 is control). Initially, Zein: GA coacervates were prepared at ratio 2:1 and at pH 4.5 ± 0.05 (by slow addition of 0.5% (w/v) glucono-δ-lactone) and kept for 1 hr at room temperature (25 °C). Flax oil and 25% Span 80 of total biopolymer concentration were mixed together. Then the former solution and the latter one were mixed together and homogenized (Brinkmann Homogenizer Polytro, San Jose, CA) at 10,000 rpm for 3 min. Homogenized mixture was kept for 1 hr at orbit shaker (VWR International, PA, USA) at 400 rpm at room temperature (25 °C). Finally, Tgase enzyme at 5% of total biopolymer concentration was added and kept at orbit shaker for 3 hr. Deionized water was used to maintain total biopolymer concentration i.e. 5% (v/v). For stability of emulsion of Zein: Ga coacervates zeta (ζ) potential was carried. Using same procedure followed in 3.1.2.

3.2.2.4. Characterization of flax oil oil-water emulsion using Zein: GA complex coacervates

Samples for photomicrographs were prepared for flax-oil emulsions at different percentages (5, 25,75), Zein and gum arabic at ratio 2:1 and deionized water was used to maintain total biopolymer 5% (v/v) at pH 4.5 ± 0.05 by slow addition of 0.5% (w/v) glucono-δ-lactone and using 5% Tgase and 25% Span 80 of total biopolymer concentration. Differential Interference Contrast (DIC) photomicrographs were obtained using same procedure followed in 3.1.4.

3.2.3. Statistical analysis

Same procedure was followed used in 3.1.6 for readings obtained in 3.2.2.1 and 3.2.2.3. For 3.2.2.2., readings obtained involved two factors; Ratio and percentage of surfactants, sample

layout was 2×2 factorial in completely randomized design. Analysis of variance was perform was using 'GLM' procedure in SAS as described in 3.1.4.

3.3. Study 3 (Formation of Zein: GA coacervates: effect temperature on their stability)

3.3.1. Turbidimetric and zeta (ζ) potential using to determine stability of Zein: GA coacervates by influence of temperature

The procedure of influence of temperature was adapted from Weinbreck and others (2004) and Cabra and others (2006).

3.3.1.1. Influence of temperature on stability of Zein: GA coacervates using turbidimetric analysis

Influence of temperature was determined by changing temperature of formed Zein: GA coacervates. Initially, mixtures of Zein and GA were prepared by mixing appropriate concentration of stock solutions, at ratio 2:1 and at pH 4.5 ± 0.05 (slow addition of 0.5% (w/v) glucono-δ-lactone). Milli-Q water was used to maintain total biopolymer concentration of 5% (v/v) (denoted as C). Samples after addition of stock solutions were kept at respective temperature using water bath (Model 10L A; VWR International, PA, USA) for 1 hr. Absorbance measurements were performed using Zein: GA mixture solution over temperature range (10, 25, 50, 75, 90 ± 2 °C), Using same procedure followed in 3.1.2.2.

3.3.1.2. Zeta (ζ) potential (mV) of Zein: GA complex coacervates at different temperature

Influence of temperature was determined by changing temperature of formed Zein: GA coacervates. Initially, mixtures of Zein and GA were prepared by mixing appropriate masses of stock solutions, at ratio 2:1 and at pH 4.5 ± 0.05 (slow addition of 0.5% (w/v) glucono-δ-

lactone). Milli-Q water was used to maintain total biopolymer concentration of 5% (v/v) (denoted as C). Samples after addition of stock solutions were kept at respective temperature using water bath for 1 hr. Zeta (ζ) potential of Zein: GA coacervates were carried. The same procedure as described in 3.1.2.3 was followed, but each measurement was taken at different temperature i.e. (10, 25, 50, 75, 90 ± 1 °C).

3.3.2. Statistical analysis

Statistical analysis procedures were the same as described in 3.1.5.

4. RESULTS AND DISCUSSION

4.1. Study 1(Formation of Zein: GA coacervates: effect of pH and biopolymer ratio on their formation and their stability)

Coacervation is based upon phenomenon of colloid formation. Complex coacervation is based upon two phase system as it forms two immiscible liquids i.e., one is formed coacervates phase and other is solvent phase. Formation of coacervates depends on pH and temperature of the mixture (Agnihotri and others 2012). In this study, influence of coacervation on pH and biopolymer ratio was predicted by analyzing turbidity over pH range (7.0 ± 0.05-2.5 ± 0.05). Absorbance (A) of formed Zein: GA coacervates was measured at 310 nm whereas ζ potential was determined to their stability (Eastman J 2010).

4.1.1. Turbidimetric analysis by using acidification of Zein: GA complex coacervates

Biopolymer ratio plays a critical for the formation of complex coacervates. Ratio of biopolymers affects the charge balance of coacervates, and overall affects the behavior of formed complexes. Factors such as pH, temperature and biopolymer mixing ratio influences the formation and stability of formed coacervation. If one of the biopolymer is in excess, soluble coacervates could be observed due to the presence of ionic charges (Ye 2008). For this study pH range 7.0 ± 0.05- 2.5 ± 0.05 was used; since pKa value of gum arabic is ~ 2.2 whereas isoelectric point (pI) value of Zein protein is ~ 6.8.

According to Ye and others (2004), as pH of the mixture (Zein and gum arabic) goes above pI value of Zein and pKa value of gum arabic, protein and polysaccharide would be protonated i.e. both structure will start to repel each other. Therefore, below or above a pH range

(in this study, 7.0 ± 0.05-2.5 ± 0.05), the electrostatic interactions or attractive forces between Zein and gum arabic will not be strong enough to form coacervates.

The study 1 determined the ratio of Zein and gum arabic for the formation of complex coacervates. Firstly, the formation of Zein: GA complex coacervates were confirmed by turbidimetric analysis. For this study, the biopolymer concentration was kept constant i.e. 5%. Basically, during this study, the effect of pH and ratio was observed by using Zein protein and gum arabic polysaccharide were mixed at different ratio with change in pH from 7.0 ± 0.05-2.5 ± 0.05.

The analysis of variance for the ratio of Zein: GA (1:1, 1:2 and 2:1) was displayed in table 1. As observed from distribution figure 1, Zein: GA ratio 1:1 and 1:2, as compared to ratio 2:1.

As observed from figure 1 (distribution graph of ratio), maximum absorbance was observed at ratio 2:1 whereas for ratio 1:1 and 1:2 were almost negligible (below 0). As for ratio 1:1, both protein and polysaccharide were at equal counterions. Low absorbance indicates less interaction occurred between gum arabic and Zein, though impact of the ratio was investigated through pH range 7.0 ± 0.05-2.5 ± 0.05. This could indicate presence of counterions of gum arabic molecules or more than Zein molecules were unable to form a charge balance coacervate.

Table 1. Ratio of Zein: GA used for complex coacervate formation

Ratio	N	Mean	
2:1	30	0.36	a
1:1	30	0.31	b
1:2	30	0.28	c

*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$

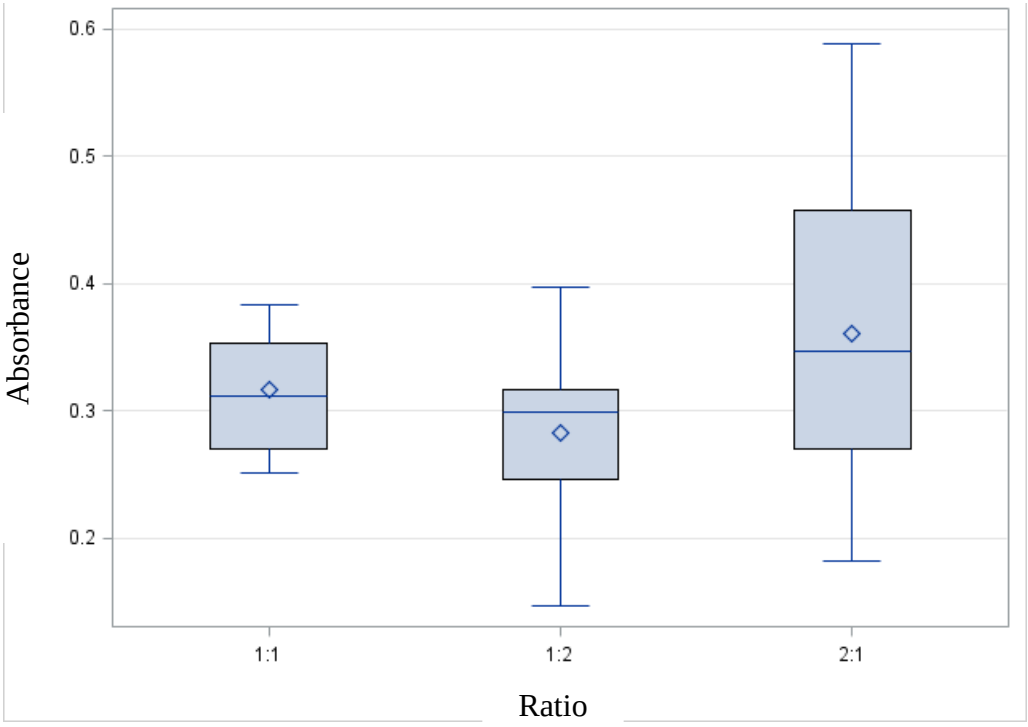

Figure 1. Distribution graph of absorbance (A) based upon ratio of Zein: GA at room temperature (Each value was obtained in triplicates).

Zein-GA complex coacervates formation is a result of electrostatic attractive forces between these two oppositely charged biopolymers under favorable conditions such as pH and protein: polysaccharide ratio (Liu and others 2010). In the mixed biopolymer system, multiple structure-forming events occurred, arising from interactions between individual GA chains and small protein aggregates. Mixtures of Zein: GA at different ratios were prepared, absorbance at

ratio 2:1 were relatively high compared to ratio 1:1 and 1:2. Therefore, further experiments were continued of Zein: GA at ratio 2:1.

Table 2. Absorbance at ratio of Zein: GA (1:1, 1:2 and 2:1) over pH range 7.0 ± 0.05-2.5 ± 0.05

pH	N	Mean	
7.0	9	0.232	f
6.5	9	0.274	e
6.0	9	0.308	d
5.5	9	0.324	cd
5.0	9	0.410	a
4.5	9	0.422	a
4.0	9	0.372	b
3.5	9	0.354	bc
3.0	9	0.272	e
2.5	9	0.229	f

*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$

Interactions between protein and anionic polysaccharide arises when they are mixed in aqueous environment are known as electrostatic interactions. From table 2., it could be predicted that at pH 4.5 ± 0.05 maximum absorbance was observed (denoted as pH_{max}) irrespective of Zein: GA ratios. Absorbance at pH 4.5 ± 0.05 and 5.0 ± 0.05 were not significantly different but when absorbance at ratio 2:1 (table 3). Absorbance values at pH 3.5 ± 0.05 and 5.5 ± 0.05 were also non-significant in both tables (2 and 3) indicates towards process of association and dissociation of coacervation.

Table 3. Absorbance of Zein: GA at 2:1 ratio over pH range 7.0 ± 0.05-2.5 ± 0.05

pH	N	Mean	
7.0	3	0.187	g
6.5	3	0.271	e
6.0	3	0.305	d
5.5	3	0.378	c
5.0	3	0.467	b
4.5	3	0.553	a
4.0	3	0.481	b
3.5	3	0.407	c
3.0	3	0.323	d
2.5	3	0.235	f

*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$

In figure 2 and table 3, absorbance was plotted against pH values. As shown, at ratio 2:1, maximum absorbance was observed at pH 4.5 ± 0.05. Absorbance values at pH 4.0 ± 0.05 and 5.0 ± 0.05 were not significantly different whereas same goes for pH values 3.5 ± 0.05 and 5.5 ± 0.05 and for 3.0 ± 0.05 and 5.0 ± 0.05. This proves that when pH < pI of Zein(~ 6.8), association phase of coacervates initiates and it completes when pH = pH_{max} and when pH < pH_{max} dissociation phase of coacervates initiates and concludes at pH close to pKa value of gum arabic.

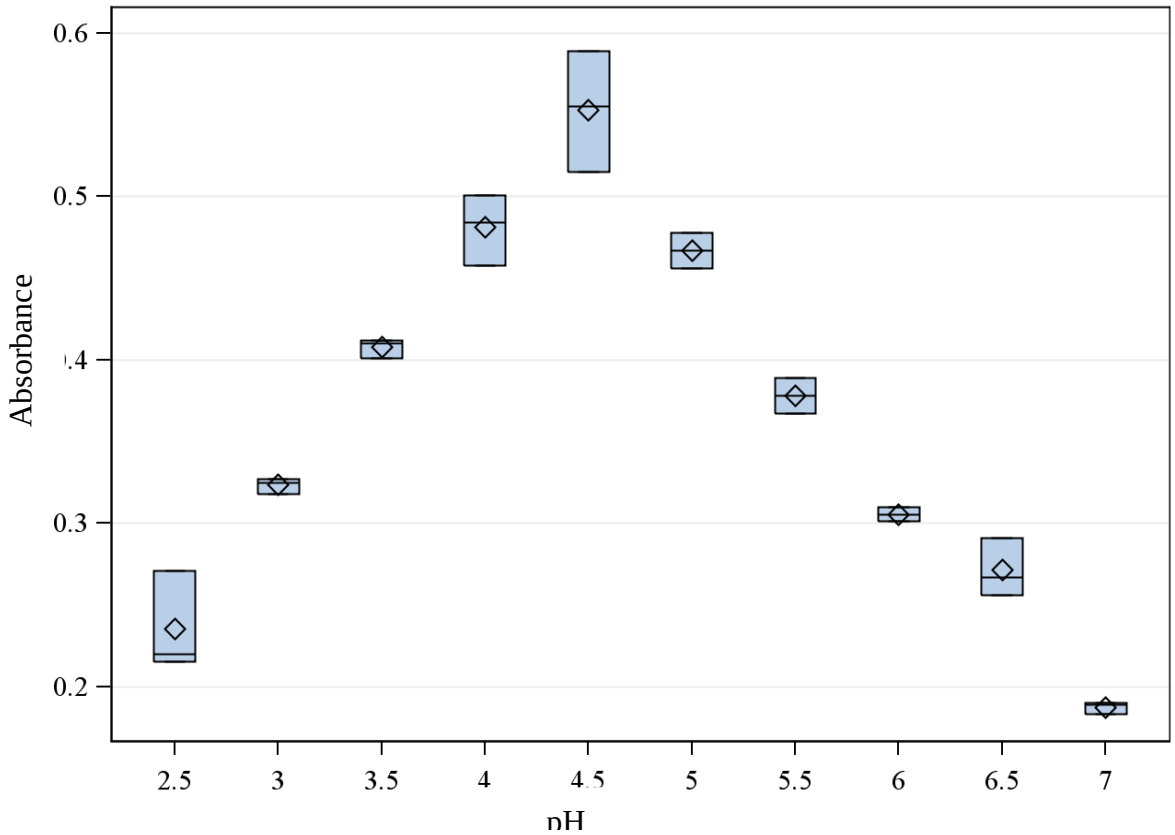

Figure 2. Distribution of absorbance of Zein: GA solution at ratio 2:1 over pH range 7.0 ± 0.05-2.5 ± 0.05 (Each value was obtained in triplicates)

In this study, interactions between Zein and the anionic polysaccharide gum arabic occurred at pH below the Zein's isoelectric point (pI) i.e. ~ 6.8, corresponding to the formation of soluble protein complexes. Under these conditions, binding sites on gum arabic includes negatively charged carboxylate groups which are attracted towards positively charged imidazole groups on Zein protein (Liu and others 2010). The presence of gum arabic prevented one of possible event i.e. Zein clustering when pH values decreases i.e. pH > 6.5 ± 0.05. This could be due to electrostatic repulsion occurring between positively charged gum arabic and positive charges groups on the surface of the Zein molecules.

Once the complexes become sufficient in size and number as indicated by sudden increase in turbidity from table 3.: the solution transitioned from being transparent to turbid

(absorbance increased) due to colloid formation formed at pH 5.0 ± 0.05. The absorbance reached a maximum at pH 4.5 ± 0.05, indicating maximum biopolymer interactions and overall charge neutralizations in mixture of biopolymers.

As solvent's pH dropped below pH_{max}, the Zein: GA coacervates began to disassociate; carboxylate groups on gum arabic carry more negative charges to due addition of glucono-delta lactic acid which decreases pH close to it pKa value (i.e. ~ 2.2). Dissociation process where pH > pH_{max} is relatively gains quickly to association where pH < pH_{max}, reason could be due to the increase in formation of protein-protein aggregates stabilized due to hydrophobic interactions between the structure. Dissolution of complex structures occurred at pH 3.0 ± 0.05 (Liu and others 2010).

4.1.2. Zeta (ζ) potential (mV) of Zein: GA complex coacervates

Table 4. Zeta (ζ) potential (mV) of Zein: GA at ratio 2:1 over pH 7.0 ± 0.05- 2.5 ± 0.05

pH	N	Mean	
7.0	3	-0.25	a
6.5	3	-22.86	c
6.0	3	-28.7	e
5.5	3	-28.66	e
5.0	3	-31.73	f
4.5	3	-32.66	g
4.0	3	-27.63	d
3.5	3	-22.53	c
3.0	3	-18.6	b
2.5	3	-0.06	a

*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$

For this study, zeta (ζ) potential of formed coacervate of Zein: GA at ratio 2:1 was observed over pH range 7.0 ± 0.05–2.5 ± 0.05. The higher ζ value (± 30 mV), higher stability of

coacervates formed. In this study, highest value observed was -32 mV, which indicates that at pH 4.5 ± 0.05 formed coacervates were highly stable. From table 4, at pH 2.5 ± 0.05 and 7 ± 0.05 zeta value were not significantly different, pH values close pKa value of gum arabic and pI value of gum arabic respectively, lower value of zeta (ζ) potential was observed, indicating less stability and more aggregation of formed complexes. Zeta (ζ) potential values at pH 3.5 ± 0.05 and 6.5 ± 0.05 were also not significantly different, indicating initiation of dissociation and association phase during coacervate formation.

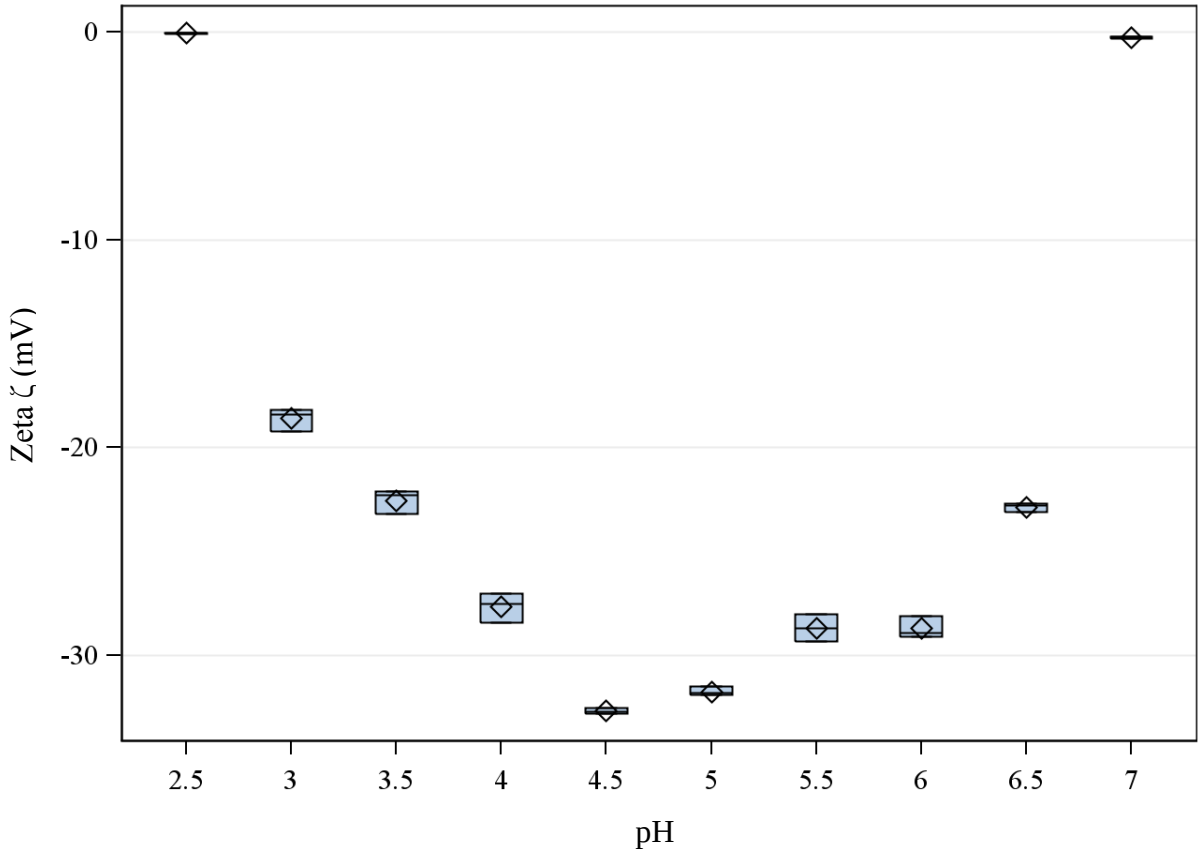

Figure 3. Distribution of zeta (ζ) potential (mV) of Zein: GA at ratio 2:1 over pH range 7.0 ± 0.05-2.5 ± 0.05 (Each value was obtained in triplicates)

According to Liu and others (2010), pH_{max} is considered to be pH range where surface charge on coacervates is neutral but zeta (ζ) potential is the measure of potential formed between surface charge and counterions, higher zeta (ζ) potential indicates that lower tendency of

particles to aggregate i.e. more stable (Eastman J 2010). From figure 3 and 4, it was evident that at pH 4.5 ± 0.05 highest ζ values was observed, indicating lower indication of aggregation, since each particle is surrounded by higher negative potential due to which strong repulsive forces were present between each formed particle, thus formation of Zein: GA complex coacervates.

4.1.3. Characterization of formed Zein: GA coacervates using microscope

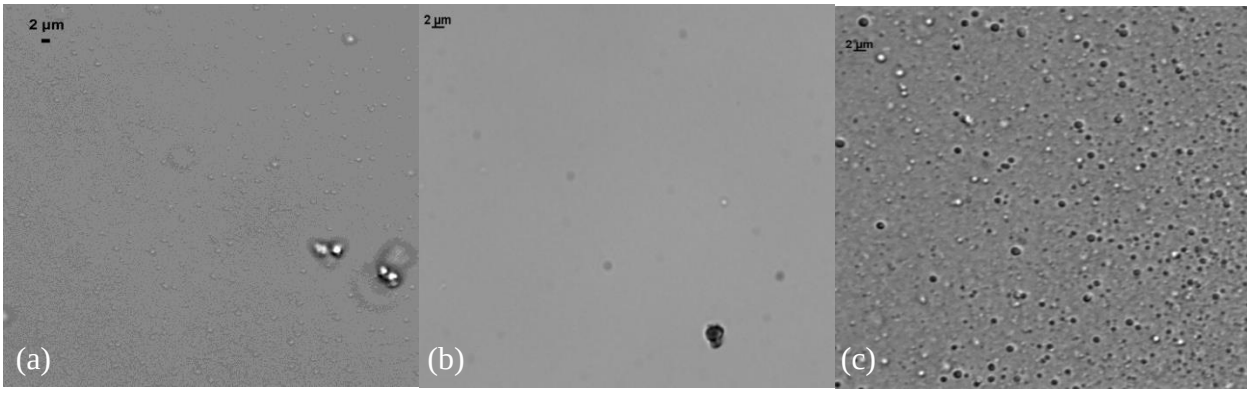

Figure 4. Microscopic images of (a) Zein solution at 4.5 ± 0.05 (b) GA solution at 4.5 ±0.05 and (c) Zein: GA coacervates at 4.5 ± 0.05

In figure 4, images were taken of Zein: GA coacervates at ratio 2:1 suspended in solvent using microscope at pH 4.5 ± 0.05. As observed, sample shown in (c) has visible formed structures which are moving in solvent as formed colloids in this system. Whereas image (a) and (b) were taken as control, Zein protein and gum arabic polysaccharide were dispersed in the solvent at same concentration at pH 4.5 ± 0.05. But sample prepared for image (a) no gum arabic was added whereas for image (b) no Zein protein was added. As observed not coacervation occurred, major particle observed in image (a) were indication of protein-protein aggregates formed due to pH change.

Thus study 1 was concluded with results showing that Zein: GA coacervates were being formed at ratio 2:1 and at pH 4.5 ± 0.05, as maximum turbidity was observed and also evident

by microscopic images (shown in figure 4). These formed coacervates were also highly stable at pH 4.5 ± 0.05, predicted by measuring zeta (ζ) potential.

4.2. Study 2 (Determination of the stability of emulsion of flax-oil formed using Zein: GA complex coacervates using transglutaminase (Tgase), Tween 80, and Span 80)

An emulsion is usually defined as a dispersion of two liquids, which are immiscible to each other. Food emulsions contain oil as one and water as another phase of the two immiscible liquids (McClements 2009). McClements (2009) described major steps for the formation of the stabilized emulsion: addition of surfactant and biopolymers mixture formation. The emulsion formation process includes three steps: pre-homogenization, homogenization and post-homogenization.

In this study, following three steps were conducted to form stabilized emulsion. Pre-homogenization into phase they are most soluble. Surfactant used was dissolved in oil, formed Zein: GA coacervates were dissolved in water and both former and later solutions were homogenized in water. For this study, Tween 80 (Polyethoxylate sorbitan monooleate) and Span 80 (Sorbitan monooleate), a non-ionic surfactants were used, in different ratios. Homogenization step involves using homogenizer to convert two immiscible phases (oil and water) into an emulsion (Walstra 1993, 2003). In this study, a high speed mixer, a type of homogenizer, was used. Post Homogenization step involves to ensure long –term quality characteristics.

4.2.1. Transglutaminase (Tgase) activity for stabilization of Zein: GA coacervates

In this study, post homogenization step during this process, tranglutaminase (Tgase) enzyme was added to stabilize the formed emulsion using Zein: GA complex coacervates formed at ratio 2:1 and pH 4.5 ± 0.05. To predict stability of protein molecules zeta (ζ) potential as the

parameter was used for this study. To predict stability of formed complex coacervates zeta (ζ) potential as the parameter was used.

Table 5. Zeta (ζ) potential (mV) of Zein: GA coacervates at different percentage of Tgase enzyme at pH 4.5 ± 0.05

Tgase (%)	N	Mean	
0	3	-32.66	d
1	3	-34.76	e
5	3	-36.7	f
10	3	-31.5	c
25	3	-28.73	b
50	3	-28.66	b
75	3	-0.10	a

*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$

For the formation of stabilized emulsion, concentration of Tgase was predicted to be used. As shown in table 5, at each percentage of Tgase zeta (ζ) potential (mV) was significantly different excluding at percentage 50% and 25%. Greater negative potential was observed at 5% Tgase of total biopolymer in aqueous phase. Zero percent Tgase is the control of this experiment. Basically, tranglutaminase (Tgase) enzyme plays a crucial role in modifying protein structure by catalyzing the transfer reaction of acyl (RCO-) group. For Zein protein, reaction occurs between ɣ-carboxylamide group of peptide-bound glutamine acts as acyl donor and a primary amine acts as acyl group acceptor (Dickinson 1997; Motoki and Segurob 1998). High percentage of Tgase addition usually results in crosslinking of protein, which could also lead towards coalescence i.e. gelling of protein molecules.

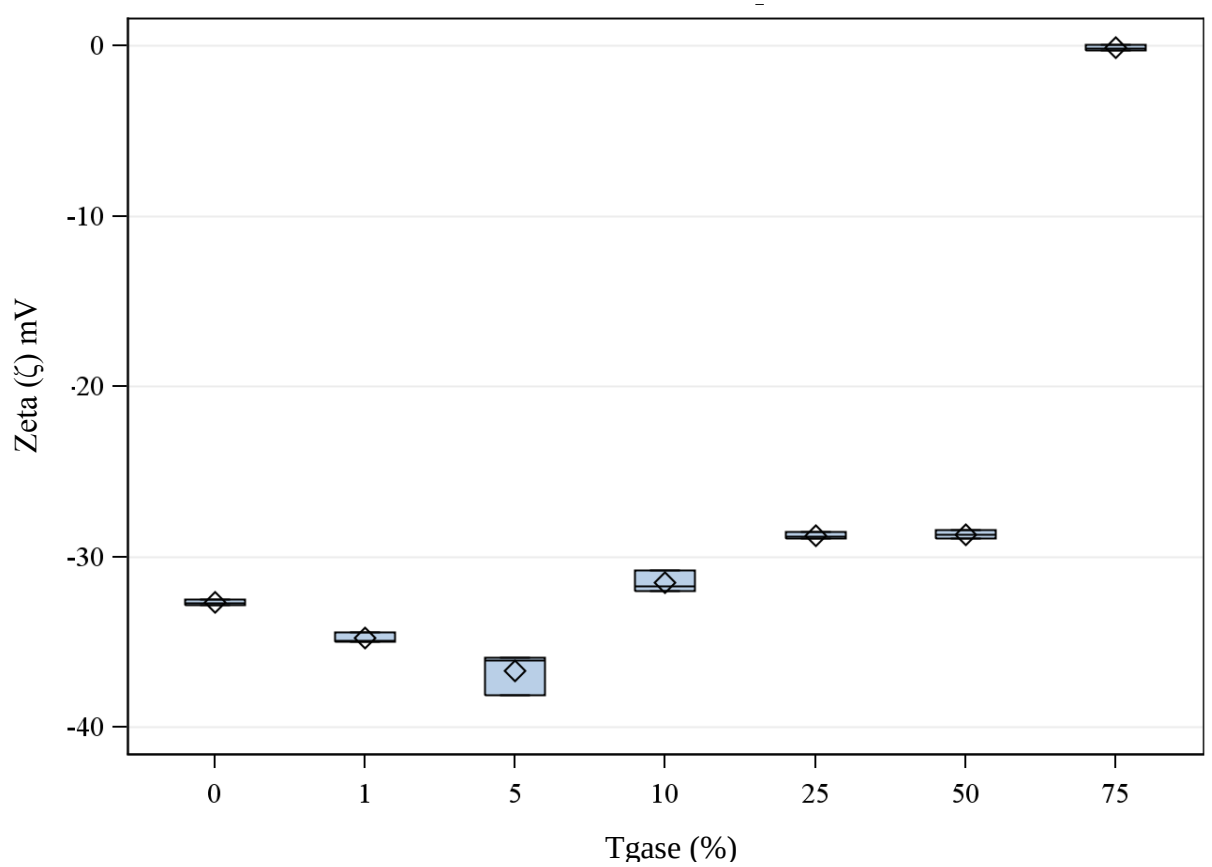

Figure 5. Distribution plot of zeta (ζ) potential against different percentage of Tgase added to Zein: GA coacervates at ratio 2:1 and pH 4.5 ± 0.05 (Each value was obtained using triplicates)

As shown in table 5, 5% Tgase of total biopolymer concentration displayed maximum stability of Zein: GA complex coacervates i.e. -36.7 mV indicating maximum stability of Zein: GA coacervates. Addition of Tgase enzyme modifies protein due to transfer of acyl group. As percentage increases, coalescence between protein molecules increases i.e. covalent bond between two protein molecules becomes stronger and further causes gel formation. As observed in figure 5, above 5% Tgase, zeta (ζ) potential decreases indicating Zein: GA coacervates are coming closer to each other. At 75% Tgase, lowest zeta potential was observed i.e. -0.10 mV.

Surfactant such as Tween 80 and Span 80 function is to form a compact absorbed layer to reduce interfacial tension between two immiscible liquids such as water and oil (Wilde 2000).

Tween 80 and Span 80 are lower molecular weight, ionic and non-ionic surfactants, respectively. They are widely used as surfactants because of their high molecular weights. In food industry, these surfactants are used with proteins, in order to provide improved stability to protein-stabilized oil-in-water formed emulsion (Bosa and Vlie 2001; Halling 1981).

For formation of stabilized emulsion, addition of surfactant is necessary during first step of the process. For this study, two surfactants were used i.e. Span 80 and Tween 80, and used at different ratios i.e. 1:0, 3:1, 1:1, 1:3 and 0:1 respectively and then stability of Zein: GA coacervates were analyzed. One of the most crucial interactions occurs between Zein: surfactants for the formation of stabilized emulsion, as higher percentage could cause destabilization of emulsion. As at higher percentage of surfactant, molecules displace protein molecules from formed interfacial interaction, in this study, displacing Zein protein from electrostatic interaction formed with gum arabic (Morris 2009).

To resist this displacement, protein usually forms linkages together into a network (McClements 2009). This displacement mechanism is not specific for any protein (flexible or globular) or any type of surfactant used (Lower molecular weight or higher molecular weight surfactant) at any interfaces (oil-water or air-water). This indicates that formed oil-water interface could be stabilized by increasing the strength of protein-protein linkages (McClements 2009; Morris and Gunning 2008).

4.2.2. Percentage of Tween 80 and Span 80 for stabilization of Zein: GA coacervates

In this study, for pre homogenization step during this process, Tween 80 and Span 80 surfactants were added to stabilize the formed emulsion using Zein: GA complex coacervates formed at ratio 2:1 and pH 4.5 ± 0.05.

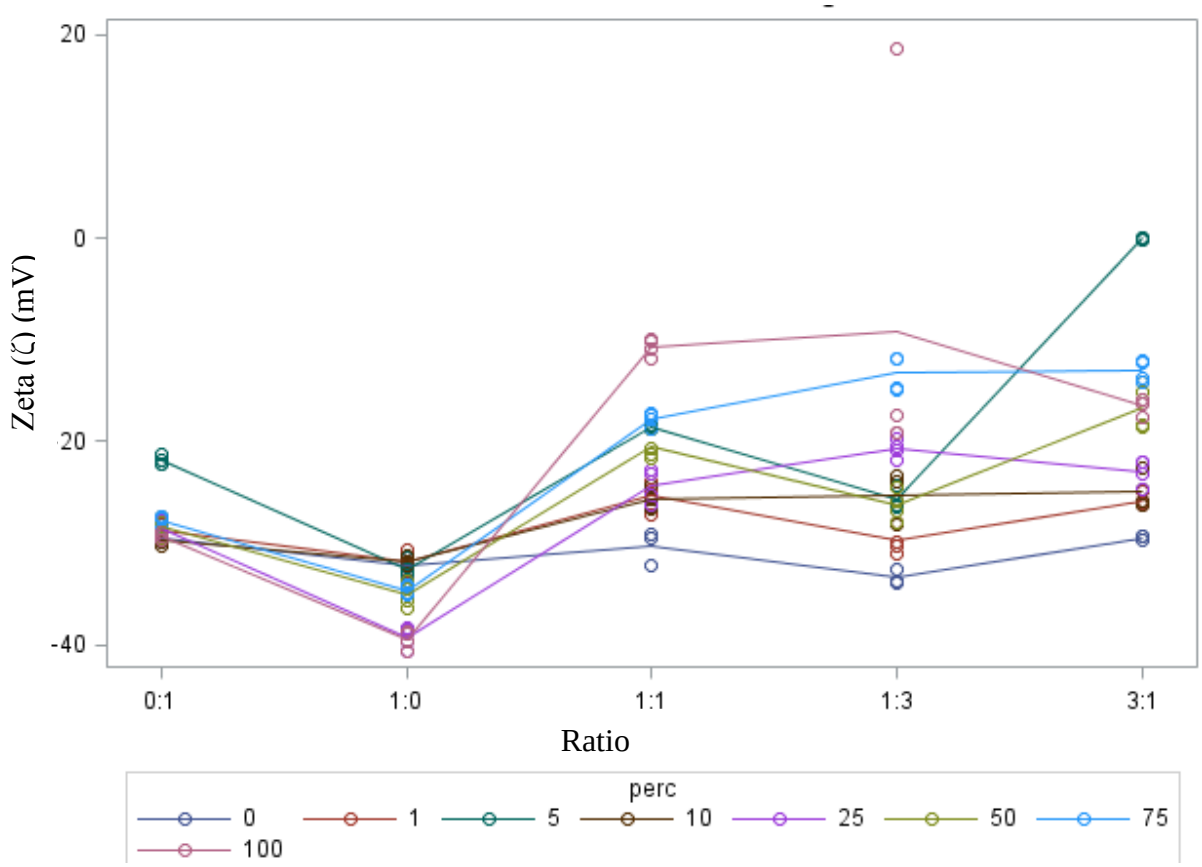

Figure 6. Comparison between different percentages of Span 80 using zeta (ζ) potential (mV) values of Zein: GA coacervates at ratio 2:1, at pH 4.5 ± 0.05 and at 5%Tgase (Each value was obtained in triplicates)

As displayed in figure 6, Span 80: Tween 80 (1:0) exhibited maximum stability in contrast to Tween 80 mixed with Span 80 at different ratios. At 25% Span 80 maximum stability i.e. ~ -40 mV was observed. Stability of formed structure was analyzed using zeta (ζ) potential as the parameter.

In this study, Tween 80 and Span 80 at different ratios respectively i.e. 1:0, 0:1, 1:3, 1:1 and 3:1 and each ratio at different concentration were investigated. In figure 6, as observed, at high percentage of surfactant lower stability was displayed, due to displacement process occurring between surfactant and protein. Displacement process increased at Span 80: Tween 80

at ratio1:1 could be due to increase number of surfactants. But it was also observed that with increase in concentration of Tween 80, lower stability was displayed by formed complexes.

4.2.3. Preparation of oil-water emulsion

In previous experiments of this study, percentage of Tgase and Span 80 were determined to form stabilized emulsion using flax-oil using Zein: GA coacervates.

Table 6. Zeta (ζ) potential (mV) of Zein: GA coacervates at different percentage of flax oil at ratio 2:1, pH 4.5 ± 0.05, 5% Tgase and 25% Span 80

Flax oil (%)	N	Mean	
0	6	-39.81	C
1	6	-45.86	D
5	6	-42.48	cd
10	6	-41.96	C
25	6	-35.6	B
50	6	-2.75	A
75	6	-0.98	A
100	6	-0.50	A

(*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$)

As observed in this experiment, at 50% Flax oil or above, zeta (ζ) potential values were not significantly different whereas at 0 (control), 1%, 5% and 10% Flax oil were also not significantly different. The most stable emulsion was formed 1% flax oil i.e. -45.86 mV. As percentage of flax oil increased, stability of emulsion started decreasing. Though Zein: GA complex coacervates were able to form stabilized emulsion up to 25% Flax oil. At 50% Flax oil destabilization of formed emulsion was observed.

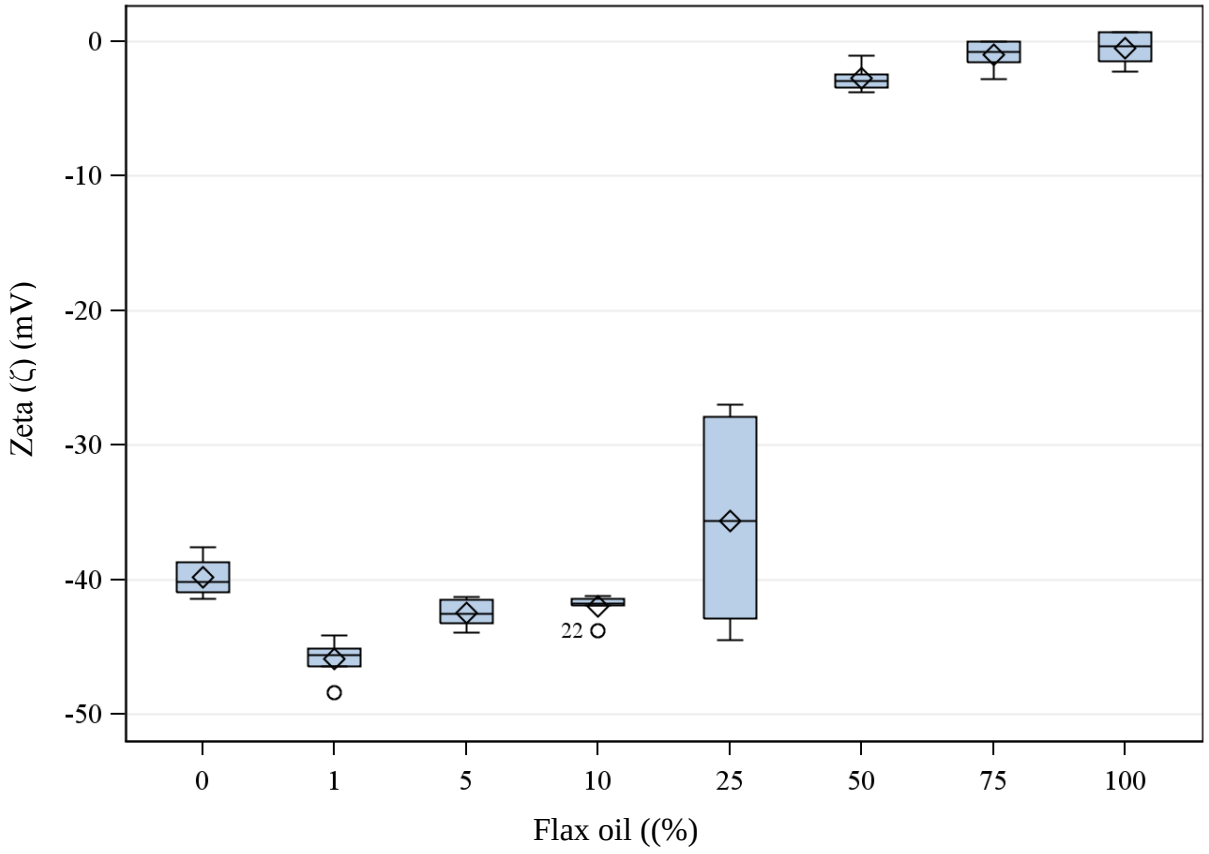

Figure 7. Distribution plot of zeta (ζ) potential (mV) against percentage of flax oil added to Zein: GA coacervates at ratio 2:1, pH 4.5 ± 0.05, at 5% Tgase enzyme and 25% Span 80 (Each value was obtained in triplicates)

From figure 7 and 8, it was evident that stabilized emulsion using Zein: GA complex coacervates were formed up to 25% flax oil. At 25% Flax oil, sample was observed to be polydispersed, as shown in figure 7 and 8. Since percentage of flax oil has increased and interfacial (water-oil) interaction has also increased, which led to destabilization of emulsion. But as percentage of Flax-oil reached at 50% destabilization could be easily observed as it goes close to 0 mV.

4.2.4 Characterization of flax oil-water emulsion using Zein: GA complex coacervates

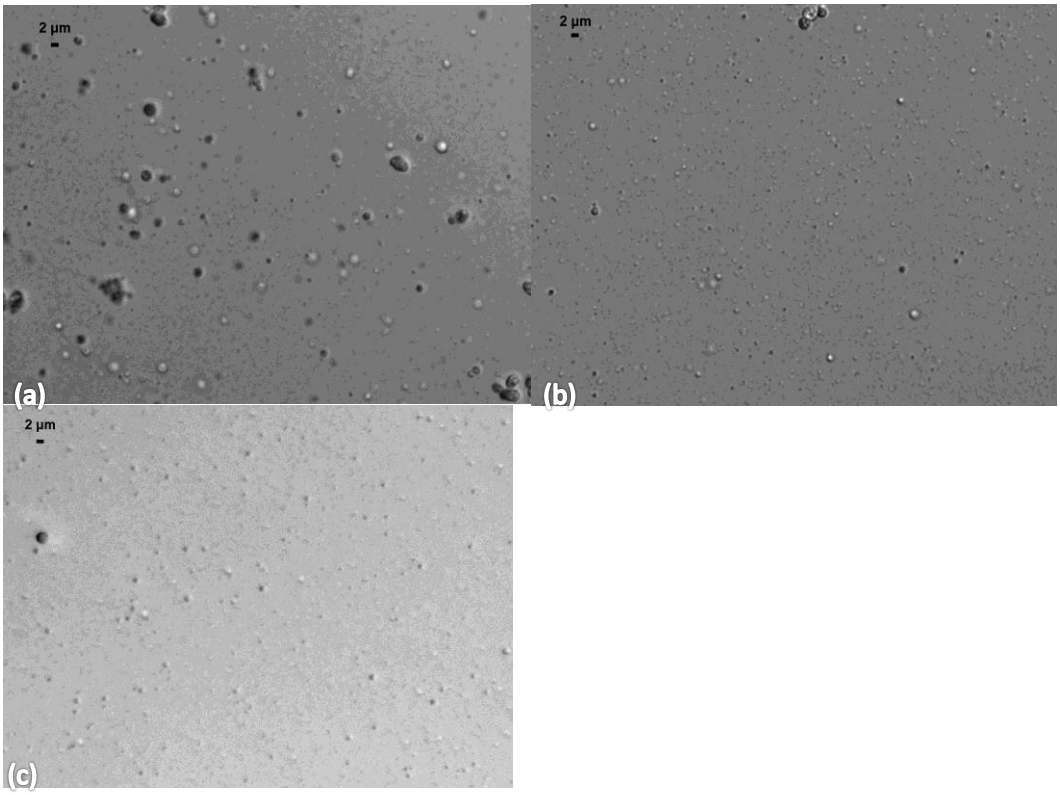

Figure 8. Microscopic images of Zein: GA complex coacervates at ratio 2:1 at pH 4.5 ± 0.05, 5% Tgase, 25% Span 80 (a) 5% flax oil, (b) 25% flax oil and (c) 75% flax oil

4.3. Study 3 (Formation of Zein: GA coacervates: effect of temperature)

Effect of temperature on Zein: GA coacervate formation was determined. Weinbreck and others 2004 investigated the effect of temperature by conducting turbidimetric titration. It was determined temperature had slight increase in pH_{max} during coacervate formation. This study observed that if another type of interaction was involved during coacervation other than electrostatic interactions, impact of temperature might have increased. Effect temperature and pH was later investigated on α-Zein using Circular dichrosim spectroscopy (CD) by Cabra and others 2006. It was concluded that increase in temperature induces change in secondary structure of Zein i.e. by formation of more disulfide cross-linked oligomers of Zein protein does leading

towards protein aggregation. Turgeon and others 2007 explained further the effect of temperature based upon the interactions. It was described as decrease in temperature favors hydrogen bonding whereas increase in temperature favors hydrophobic interactions.

In this study, influence of temperature factor on stability of formed Zein: GA coacervates was determined. Firstly, turbidimetric analysis was conducted followed by zeta (ζ) potential analysis using pH_{max} i.e. 4.5 ± 0.05.

4.3.1. Influence of temperature on stability of Zein: GA coacervates using turbidimetric analysis

Table 7. Turbidimetric analysis of Zein: GA complex coacervates at ratio 2:1 at pH 4.5 ± 0.05 at different temperature

Temp	N	Mean	
10	3	0.501	b
25	3	0.649	a
50	3	0.341	c
75	3	0.372	C
90	3	0.366	C

(*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$)

In table 7, maximum absorbance was observed at 25 ± 2 °C, whereas no significant difference was observed in absorbance at temperature 50, 75 and 90 ± 2 °C. But as observed at 10 ± 2 °C, absorbance close to 0.5 was observed. But this turbidimetric analysis does not predict the stability of Zein: GA coacervates.

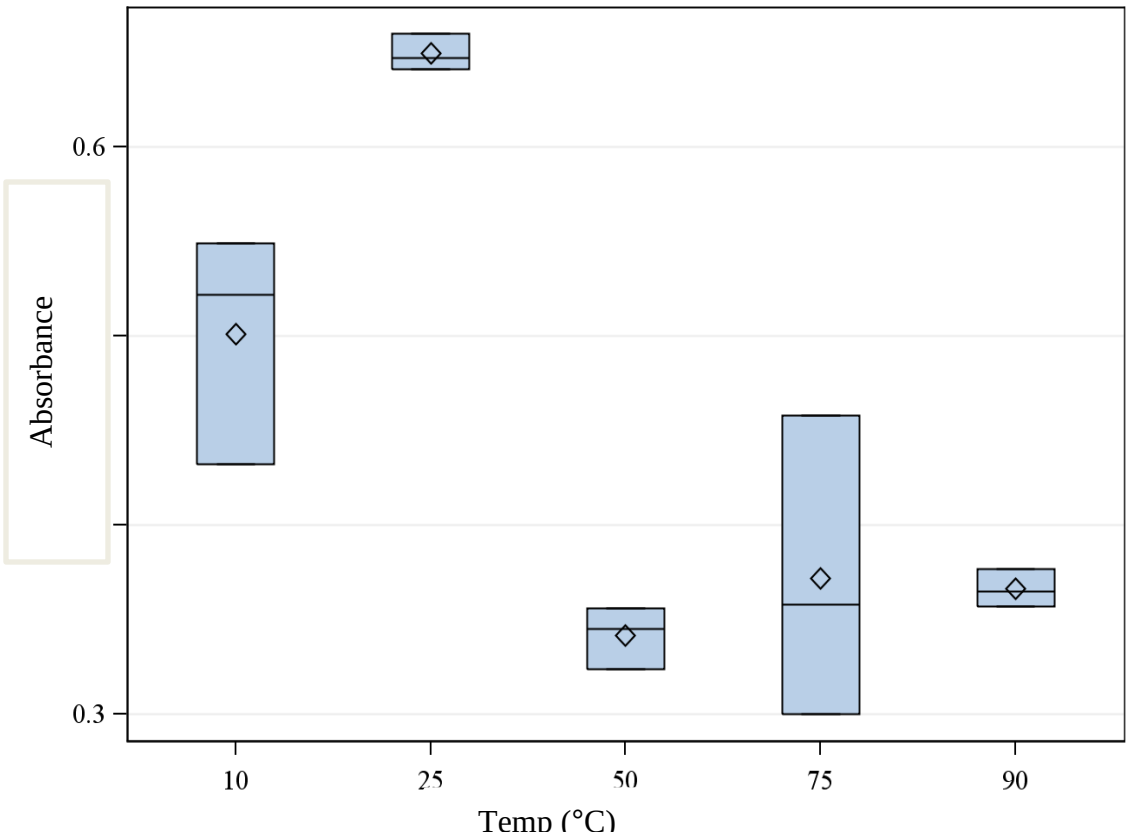

Figure 9. Distribution of absorbance (A) against various temperature of Zein: GA complex coacervates at ratio 2:1 at pH 4.5 ± 0.05 (Each value was obtained using triplicates)

As shown in figure 9, maximum absorbance was observed at 25 ± 2 °C and after increasing temperature absorbance decreases. This indicates less formation of Zein: GA coacervates. Due to increase in temperature, Zein's secondary structure modification leads to protein-protein aggregation. At lower temperature, hydrogen bonds formation is more favored. Increase in temperature also leads towards conformational changes in gum arabic, which may lead to change in it functional properties.

4.3.2. Zeta (ζ) potential (mV) of Zein: GA complex coacervates at different temperature

Table 8. Zeta (ζ) potential (mV) of Zein: GA complex coacervates at ratio 2:1 at pH 4.5 ± 0.05 at different temperature

Temp	N	Mean	
10	3	-27.26	c
25	3	-34.1	e
50	3	-29.63	d
75	3	5.28	a
90	3	0.13	b

(*Each value is the mean of triplicates. Different letters on column imply statistically significant differences at $p<0.05$)

In table 8, maximum zeta (ζ) potential was observed at 25 ± 2 °C i.e. -34.1 mV. Each zeta (ζ) potential value at each temperature was significantly different. At three temperatures 25 °C, 50 ± 2 °C and 10 °C were highly stable, confirming the formation of stabilized hydrogen bond formation. The least stability was observed at 75 ± 2 °C, indicating possible hydrophobic interaction.

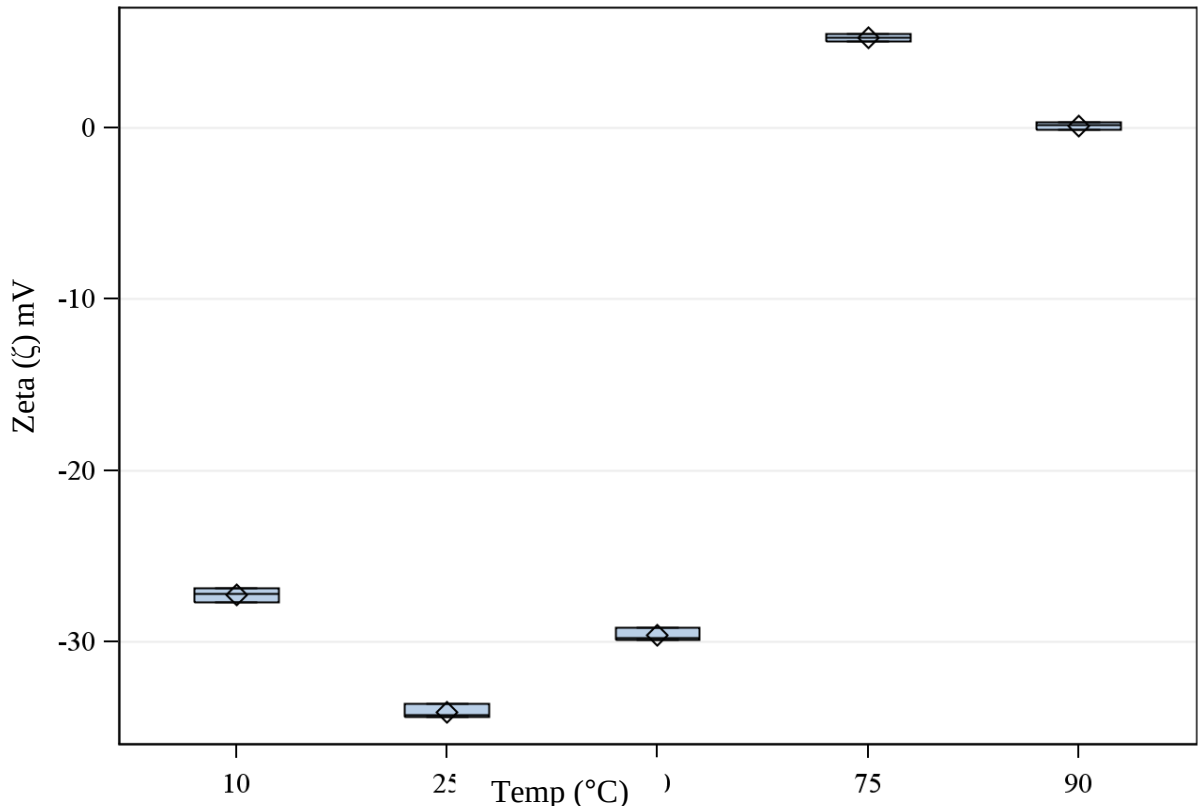

Figure 10. Distribution of zeta (ζ) potential (mV) against various temperature of Zein: GA complex coacervates at ratio 2:1 at pH 4.5 ± 0.05 (Each value was obtained using triplicates)

Figure 10, confirms that Zein: GA complex coacervates are highly stable at 25 ± 2 °C due to formed electrostatic interactions and hydrogen bond formation.

5. CONCLUSION AND FUTURE DIRECTIONS

Study 1, the optimum ratio and pH (pH_{max}) for the formation of stable Zein: GA complex coacervates were determined. Initially, optimum Zein and gum Arabic ratios for coacervate formation were investigated at different polymer ratios and pH. Turbidimetric analysis showed, at pH 4.5 ± 0.05 the maximum absorbance using Zein: GA at ratio 2:1. Zeta (ζ) potential analysis displayed similar results i.e. formation of stable Zein: GA complex coacervates at pH 4.5 ± 0.05. Microscopic images also confirmed the formed coacervates.

Study 2, the stability of Zein: GA coacervates as an emulsifier using flax oil was determined. Firstly, the concentration of tranglutaminase (Tgase) was determined at 5% of total biopolymer concentration. Zeta (ζ) potential analysis showed that at higher concentration cause coalescence of the stabilized particles. Secondly, suitable concentration to form stable emulsion using Span 80 and Tween 80 was also determined. Again zeta (ζ) potential analysis was conducted as at higher concentrations surfactants displace protein from interface (oil –water). The overall results suggest, Zein: GA complex coacervates using transglutaminase and Span 80 (surfactant) can form stable emulsion up to 25% flax oil of total biopolymer concentration.

Study 3, the optimum temperature for the stability of formed Zein: GA coacervates was determined. Turbidimetric analysis was conducted from 10-90 ± 2 °C range. Maximum absorbance was observed at 25 ± 2 °C and then at 10 ± 2 °C, but absorbance from temperature 50-90 ± 2 °C was not significantly different. Then, Zeta (ζ) potential analysis confirmed similar results: maximum stability at 25 °C, but minimum stability was observed at 75 ± 2 °C.

The present study was a baseline to identify the optimum conditions for the formation and stability of Zein: GA complex coacervates and improved emulsifying properties of Zein protein with gum arabic. These formed coacervates could be potential encapsulation materials for flavor or drug in the food industry. Further research using Zein: GA coacervates with flavor or drug molecule show potential applications for food and pharmaceutical industries.

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