Effects of Xanthan/Locust Bean Gum Mixtures on the Physicochemical Properties and Oxidative Stability of Whey Protein Stabilized Oil-In-Water Emulsions

Goutham Puli
Western Kentucky University, goutham.puli433@topper.wku.edu

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EFFECTS OF XANTHAN/LOCUST BEAN GUM MIXTURES ON THE PHYSICOCHEMICAL PROPERTIES AND OXIDATIVE STABILITY OF WHEY PROTEIN STABILIZED OIL-IN-WATER EMULSIONS

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Master of Science

By
Goutham Puli

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EFFECTS OF XANTHAN/LOCUST BEAN GUM MIXTURES ON THE PHYSICOCHEMICAL PROPERTIES AND OXIDATIVE STABILITY OF WHEY PROTEIN STABILIZED OIL-IN-WATER EMULSIONS

Date Recommended 05/03/2013

Dr. John Khourieh, Director of Thesis

Dr. Kevin Williams

Dr. Darwin Dahl

Dean, Graduate Studies and Research  Date
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Scientific evidence shows that dietary intake of the omega-3 polyunsaturated fatty acids (ω-3 PUFA) is beneficial to human health. Fish oil is a rich source of omega-3 fatty acids. However, fish oil with high levels of omega-3 PUFA is very susceptible to oxidative deterioration during storage. The objective of this study was to investigate the effect of xanthan gum (XG)-locust bean gum (LBG) mixtures on the physicochemical properties of whey protein isolate (WPI) stabilized oil-in-water (O/W) emulsions containing 20% v/v menhaden oil. The O/W emulsions containing XG/LBG mixtures were compared to emulsions with either XG or LBG alone. The emulsions were prepared using a sonicator by first mixing menhaden oil into the WPI solution and then either XG, LBG or XG/LBG mixtures were added. WPI solution (2 wt%) and gum solutions (0.0, 0.05, 0.1, 0.15, 0.2 and 0.5 wt%) were prepared separately by dissolving measured quantities of WPI in distilled water. XG and LBG gums were blended in a synergistic ratios of 50:50 for the mixture. The emulsions were evaluated for apparent viscosity, microstructure, creaming stability and oxidative stability. Addition of 0.15, 0.2 and 0.5 wt% XG/LBG mixtures greatly decreased the creaming of the emulsion. The emulsion with 0.15, 0.2 and 0.5 wt% XG/LBG mixtures showed no visible serum separation during 15 d of storage. The apparent viscosity of the emulsions containing XG/LBG mixtures was significantly higher (p < 0.05) than the emulsions containing either XG or LBG.
alone. The viscosity was sharply enhanced at higher concentrations of XG/LBG mixtures. Microstructure images showed depletion flocculation for LBG (0.05-0.5 wt%), XG (0.05-0.2 wt%) and XG/LBG mixtures (0.05 and 0.1 wt%) emulsions. Flocculation was decreased with the increased biopolymer concentration in the emulsion. The decrease in flocculation was much pronounced for the emulsion containing XG/LBG mixtures. The rate of lipid oxidation for 8 week storage was significantly (p < 0.05) lower in emulsions containing XG/LBG mixtures than in emulsions containing either of the biopolymer alone. The results suggested that the addition of XG/LBG mixtures greatly enhanced the creaming and oxidative stability of the WPI-stabilized menhaden O/W emulsion as compared to either XG or LBG alone.
Chapter 1- Introduction

1.1. Background

Food proteins and polysaccharides play a key role in the structure and stabilization of food systems through their gelling, thickening and surface-stabilizing functional properties (Tolstoguzov 1991). During manufacturing, intrinsic properties of individual components, as well as interactions between the different components, determine the final structure, texture and stability of food materials. Because texture and stability are major criteria of food quality, scientists and the food industry are primarily concerned in identifying such interactions to provide optimum food quality, design new and attractive food, and food ingredients structure (Schmitt and others 1998). The two main types of food biopolymers found in oil-in-water emulsions are proteins and polysaccharides, and some food emulsions are products containing both types of these macromolecules (Damodaran 1997). Proteins and polysaccharides have received greater attention in the food industry because of their key role in functional properties which make them recognized for excellent emulsification properties, flavor encapsulation, and high nutritional values (Charve and Reineccius 2009).

Omega-3 PUFAs, especially long-chain eicosapentaenoic and docosahexaenoic fatty acids, exert a strong positive influence on human health. At present, fish oil is the major source of omega-3 fatty acids. Eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6, ω-3) have been reported to protect against a wide range of diseases, including atherosclerosis, myocardial infarction, cancer, autism, and sudden death (Lee and others 2003; Mori 2004). However, omega-3 fatty acids are susceptible to oxidative deterioration, limiting their use in foods because of flavor...
degradation by oxidation. To utilize nutritionally beneficial omega-3 fatty acids in foods, their oxidative stability must be increased. Protein-polysaccharide complexes both at the interface of emulsion droplets and in the continuous phase of oil-in-water emulsions can inhibit lipid oxidation. Because they can be used as emulsifiers to produce physically stable oil-in-water emulsions while simultaneously inhibiting lipid oxidation, these multifunctional ingredients could be used to aid the incorporation of oxidatively unstable lipids into foods (Habibollah 2004).

When mixing proteins with polysaccharides, the interactions can be segregative or associative in nature. A homogenous mixture of co-soluble polymers can be obtained only under very specific conditions of concentration and polymer ratios. Depending on the type of the polymer, the concentration of the polysaccharide present and the environmental conditions of the solution the protein-polysaccharide interactions can improve the stability (Milena and others 2011). Emulsions have been successfully used to stabilize the omega-3 fatty acids in order to prevent lipid oxidation. Stable emulsions can be formed by adding emulsifiers, and thickening agents (proteins and polysaccharides) to overcome the activation energy of the system. These agents help in lowering the surface tension and preventing droplet aggregation (Sun and others 2007).

Whey protein isolate (WPI) is a surface active globular protein containing cysteyl residues, disulfide bonds and thiol functional groups which inhibit lipid oxidation and thus act as an antioxidant system (Gentes and others 2011; Sun and others 2007). Xanthan gum (XG) is an anionic polysaccharide obtained from bacterium, Xanthomonas campestris. Locust bean gum (LBG) is a nonanionic polysaccharide obtained from the endosperm of the seeds of Ceretonia siliqua. A synergistic interaction occurs between
XG and galactomannans such as guar gum, LBG and cassia gum. Several studies suggested that XG interacts with galactomannans to form mixed gels with high viscosity at low total polysaccharide concentrations (Tako and others 1984), and this interaction is more pronounced with LBG than with any other polysaccharide or galactomannan (Dea and others 1977). The interaction between XG and LBG is largely exploited in food applications in which thickening or gelling is desired. A considerable number of research studies have been published to elucidate the mechanisms behind XG and LBG interactions.

1.2. Thesis hypothesis

The effects of the synergistic interaction between XG and LBG combined with a protein on the oxidative stability of oil-in-water emulsions have never been investigated. The main hypothesis of this research was that the synergistic interaction between XG and LBG will enhance the stability of omega-3 fatty acids emulsions better than using either gum alone. To assess the effectiveness of synergistic interaction of XG/LBG mixtures on the oxidative stability of omega-3 PUFAs, the control is omega-3 PUFAs mixed with WPI and individual hydrocolloid gums. The hydrocolloid gum is added separately in synergistic ratios.

1.3. Thesis and objectives

1. Investigate the effect of the synergistic interaction between xanthan gum (XG) and locust bean gum (LBG) on the stability of oil-in-water emulsions.
2. Determine the optimum levels of XG/LBG mixtures that allow incorporation of the omega-3 PUFAs into emulsions and enhance the oxidative stability of omega-3 PUFAs.

3. Characterize the oxidative stability and microstructural properties of the encapsulated omega-3 PUFAs.
Chapter 2- Literature review

2.1. Omega-3 fatty acids

2.1.1. Introduction

Lipids are important nutrients. They are important for use, storage and transport of energy, for insulation and mechanical protection. In addition, lipids provide us with PUFAs. The two important PUFAs are omega-3 fatty acids and omega-6 fatty acids (Connor 1978). They are essential because they cannot be synthesized by the body and must come from food. These essential nutrients are important for several cellular functions in the body, including acting as ligands for transcription factors, precursors of signal molecules, and building blocks in the cells of the body (Drevon 1993).

Excess intake of saturated fatty acids, trans fat, and cholesterol may result in cardiovascular diseases; whereas intake of long chain omega-3 fatty acids and monounsaturated fatty acids may provide beneficial effects. Fish and other marine animals and oils from these sources are rich in omega-3 fatty acids, and they have been important ingredients of human diet for many populations during thousands of years. Consuming marine foods not only provide with healthy omega-3 fatty acids, but also replace the unhealthy nutrients like hard fat with marine fat (Drevon 2009).

The simplest omega-3 fatty acid is α-linolenic acid. In plants α-linolenic acid is synthesized from α-linoleic acid by desaturation. This desaturation reaction is catalyzed by the delta-15 desaturase enzyme. Animals and humans do not possess this enzyme, but can metabolize α-linoleic acid by further desaturation and elongation to form eicosapentanoic acid (EPA), docosahexanoic acid (DHA), and docosapentanoic acid (DPA) (Calder 2009).
2.1.2 Sources and recommended intake

Green leaves contain large amounts of their fatty acids as \( \alpha \)-linolenic acid, but they are not rich sources of fat. Several seeds, seed oils and some nuts contain significant amounts of \( \alpha \)-linolenic acid. Linseeds (flaxseeds) and their oil typically contain 45-55\% of fatty acids as \( \alpha \)-linolenic acid, whereas soya bean oil contains 5-10\% of fatty acids as \( \alpha \)-linolenic acid. Rapeseed oil and walnuts also contain \( \alpha \)-linolenic acid. Corn oil, sunflower oil, and safflower oil contain very little \( \alpha \)-linolenic acid (Burdge 2006).

Fish and other sea foods are good sources of the very long chain omega-3 fatty acids rich in DPA, EPA and DHA. Different types of the fish contain different amounts of these acids and different ratios of EPA to DHA. Fish oil is rich in very long chain omega-3 fatty acids. Different oily fish contain different amounts of the omega-3 fatty acids, different fish oils also contain different amounts of omega-3 fatty acids. A typical preparation of fish oil contains about 30\% of EPA and DHA. This means that 1 g of fish oil can provide us with 0.3 g of EPA plus DHA. The most important source of omega-3 fatty acids is \( \alpha \)-linolenic acid which is mostly found in vegetable oils. Linseed oil, canola oil and soybean oil contain approximately 57\%, 8\% and 7\% \( \alpha \)-linolenic acid respectively, but these oils are without any EPA and DHA (Racine and Deckelalbaum 2007).

Significant amounts of very long chain omega-3 fatty acids are found in fatty fishes (herring, mackerel, salmon, trout, eel, anchovies, sardines). The omega-3 fatty acids in fatty fishes or cod liver are not synthesized in the fish itself, but in very small organisms called phytoplanktons. These marine fatty acids are transferred to the respective fishes, seals and whales through the food chain. Cod liver oil is traditionally used as a source of very long chain omega-3 fatty acids. Recently krill oil is used as a
major source of marine oils. In this oil a substantial proportion of fatty acids are bound in phospholipids. In most other marine oils the majority of omega-3 fatty acids are found in triglycerides (Dyerberg 1989).

α-linolenic acid, the precursor of EPA and DHA was first isolated from hemp seed oil in 1887. In plants, leaf lipids usually contain large proportions of α-linolenic acid. This fatty acid is an important component of chloroplast membrane polar lipids. Mammals who feed on these plants convert α-linolenic acid to long chain omega-3 fatty acids EPA and DHA (Drevon 2009).

Wild animals and birds feed on these plants, but plants are very lean and contain a fat content of only 3.9% and approximately five fold more polyunsaturated fat per g than is found in domestic livestock. 4% of the fat of the wild animals contains EPA, but domestic animals contain less, or undetectable amounts of omega-3 fatty acids because they are fed with grains which are rich in omega-6 fatty acids, and poor in omega-3 fatty acids (Harris 2008). Lipids of liverworts, ferns, algae, and mosses contain α-linolenic acid, EPA and DHA. Thus both plants and animals feeding on these will be good sources of omega-3 fatty acids for human consumption. Purslane is a good non-aquatic source of α-linolenic acid. It is a wild growing plant and is a richer source of omega-3 fatty acids than any leafy vegetable (Simopoulos 2002).

Cold water fish are the major sources of EPA and DHA. These also show marked variability in their EPA and DHA content. Cold water fish obtain their EPA and DHA fatty acids by consuming α-linolenic acid (ALA) containing krill and algae. This ALA is further converted to longer and more unsaturated EPA and DHA. Seasonal variations and geographical areas may also influence the amounts of EPA and DHA in a single species.
of fish. These variations may be as much as 1.5 fold. Marine microalgae that produce triglycerides rich in DHA can be selectively cultivated to provide a continuous supply of omega-3 fatty acids with consistent levels of quality and context, but this approach involves high production costs. Krill have high amounts of EPA and DHA, but their production costs are also high. Therefore, it is better to cultivate stearidonic acid (SDA)-enriched plants because growing them is a cost-effective approach, and they are a good source of bioactive omega-3 fatty acids. Due to the increased recommendations for omega-3 fatty acid intake, the current supply of the cold water fish is insufficient to the European and North American populations. Thus SDA plants are now considered as one of the major sources of omega-3 fatty acid intake, joining ALA, EPA and DHA (Deckelbaum and Torrejon 2012). SDA is long chain PUFA. It is a metabolic intermediate in the conversion of ALA to EPA, and finally to DHA. The biological actions of SDA are similar to those of EPA and DHA. Thus SDA can become a valuable tool in meeting the current recommended intakes for long chain PUFAs (Racine and Deckelbaum 2007; Whelan 2008).

One of the major dietary sources of SDA is seafood. SDA contributes only 0.5-2% of the total fatty acids present in fish and other seafood. In addition, mackerel also contains as much as 7% of SDA (Passi 2002). Seaweed (Undaria pinnatifida) also contains SDA (0.7-1.9 mg/g dry weight). Plants from boraginaceae, grossulariaceae, caryophyllaceae, and primulaceae are unique because of their SDA contents. Echium oil is obtained from the plants of family boraginaceae. This oil is the richest commonly available plant source of SDA (3.5-9.0%) (Frankel 2002). Leaf lipids of certain types of
flowering plants from the caryophyllaceae, primulaceae, and boraginaceae families may have the highest concentration of SDA, with levels as high as 21% (Whelan 2008).

The American heart association (AHA) has recommended that healthy adults should eat at least two servings of fish per week to increase omega-3 fatty acid intake. Eating 2-4 ounces will generally provide about 1 g of omega-3 fatty acids. Another food source is omega egg, which is rich in omega-3 fatty acids. These eggs look like common eggs, but have six times the omega-3 fatty acids than conventional eggs. They also contain less saturated fat, and cholesterol than conventional eggs.

The American health association has recommended that a healthy person should get at least 5 – 10% of calories from omega-6 fatty acids in combination with α-linolenic acid, and other dietary recommendations. Dietary reference intakes (DRIs) for macronutrients were set by the institute of medicine of the national academies in 2002. An adequate intake (AI, an intake associated with a low prevalence of inadequacy) and an acceptable macronutrient distribution range (AMDR) were set for omega-3 fatty acids. An AI was set for ALA as 1.6 g/d for men, and 1.1 g/d for women aged 19-50 y. In addition, up to 10% of the AI for ALA can be provided by EPA or DHA.

The dietary guidelines for Americans 2005 report states that approximately two servings of fish per week (approximately 227 g; 8 ounces total) may reduce the risk of mortality from CHD, and that consuming EPA and DHA may reduce the risk of mortality from CVD in people who have already experienced a cardiac event (Gebauer 2006).
2.1.3. Chemical structure

Fatty acids are hydrocarbons with a carboxyl group at one end, and a methyl group at another end. Fatty acid chain lengths vary from 2-30 or more carbons, and may contain double bonds. Fatty acids containing double bonds are referred to as unsaturated fatty acids. The PUFAs are denoted by short hand nomenclature that denotes the number of carbon atoms in the chain, the number of double bonds, and the position of the first double bond with respect to methyl (ω; sometimes called n) carbon.

For example, the simplest omega-3 fatty acid ALA is denoted as (18:3 ω-3). In plants α-linolenic acid is synthesized from α-linoleic acid by desaturation. This reaction is catalyzed by delta-15 desaturase enzyme. Though humans do not possess this enzyme, but metabolize ALA by further desaturation and elongation. ALA can be converted into stearidonic acid by delta-6 desaturase enzyme. This stearidonic acid can be further elongated to 20:4 ω-3, and this fatty acid is further desaturated by delta-5 desaturase to yield EPA (20:5 ω-3). Further addition of 2 carbons to EPA yields DPA (22:5 ω-3) (Fig 2.1) (Calder 2009).
Fig 2.1. Pathway of conversion of α-linolenic acid to longer chain, more unsaturated ω-3 fatty acids (Calder 2009).

2.1.4. Health benefits

The omega-3 fatty acids have important roles relating to human health and disease. In infancy, they improve cognitive development, and learning as well as visual
development. The long-chain EPA and DHA may be beneficial in terms of decreasing the risk of depression and suicide. These fatty acids decrease the risk of certain forms of cancer (Halvorsen and Blomhoff 2011).

A number of studies report that omega-3 fatty acids are helpful in reducing the risk of stroke and coronary heart disease. Omega-3 fatty acids provide with antithrombotic, anti-inflammatory, and anti-hypertensive effects (Awad and others 2009). Thus, they reduce the risk of death due to arrhythmias. They also reduce the triglyceride levels. A dietary intake of omega-3 fatty acids has a protective effect against stroke, but this effect is weaker than those observed with cardiac morbidity and mortality (Deckelbaum and Torrejon 2012). Larger intake of fish oil supplements reduced the triglyceride levels to about 10-33%. Omega-3 fatty acids were found to have a little beneficial effect on blood pressure and coronary artery restenosis after angioplasty. Consumption of omega-3 fatty acids, fish and fish oil reduces the all-cause mortality, and various CVD outcomes such as sudden death, cardiac death, and myocardial infarction.

2.1.5. Menhaden oil

Menhaden is an abundant fish species in the United States, but it is not consumed as a human food. Menhaden is mainly considered as reduction fishery with most of the menhaden used for producing fish oil, fish meal and fish soluble protein (Yuting and others 2011). Menhaden contain large amounts of highly unsaturated fatty acids that are prone to rapid oxidation, and have a reputation for rapid spoilage. It has been shown that if menhaden are chilled rapidly and held at about 0 °C they can produce good food products in appropriate product forms (Hale and Bauersfeld 1978). Menhaden oil is rich
in eicosapentaenoic (12.8% to 15.4%) and docosahexaenoic (6.9% to 9.1%) acids, and the primary market for the oil is in aquaculture feeds. To a lesser extent, menhaden is also considered to be a bait fishery. The end products from menhaden reduction processes are fertilizers, pet foods, and feed supplements for the poultry, swine and cattle industries. Furthermore, menhaden are utilized for feeds in the catfish, trout, salmon, and shrimp aquaculture industries (Huaixia and others 2010).

2.2. Lipid oxidation

The above stated information confirms that intake of fish or fish oil decreases the mortality and morbidity, as well as risk factors of CVD. This is due to the presence of EPA and DHA in fish oils. Even though PUFAs show protective effects against CVD, negative health effects may result from the ingestion of oxidized lipids. Sometimes intake of omega-3 fatty acids does not show protective effects on CVD. These effects may be due to intake of oxidized omega-3 fatty acids. In most studies, the oxidative status of the fish oil is not determined.

Research studies on consumption levels of fish oil in different populations were shown below the level to provide beneficial health to the consumers. The major challenge in the pharmaceutical, and food industries is to develop products fortified with omega-3 fatty acids as they are highly susceptible to oxidative deterioration, thereby limiting to produce toxic free and flavored products. Therefore, functionally effective methods need to be developed to encapsulate fatty acids, and retain their original physiological functions (McClements and Decker 2010).
Lipid oxidation of fish oil and other PUFAs rich foods is a serious problem that leads to loss of shelf-life, consumer acceptability, functionality, nutritional value, and safety. PUFAs oxidation affects the quality and nutritional value of foods. Consequently, the presences of fatty acid oxidation products in human foods, especially the aldehydes, have been implicated in aging, mutagenesis, and carcinogenesis. The toxicity of these aldehydes including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) is due to their ability to crosslink to proteins and bind covalently to nucleic acids.

Lipid oxidation is a general term that is used to describe a complex sequence of chemical changes that result from interaction of lipids with oxygen-active species (McClements and Decker 2000). Access to oxygen and light, surface area, heating, and irradiation accelerate lipid oxidation, decreasing stability, and shelf-life of products containing fish oil. However, the low oxidative stability of polyunsaturated marine omega-3 fatty acids calls for effective antioxidant protection to avoid oxidative deterioration, and off-flavor development of such oil enriched foods (Taneja and Singh 2012). Lipid oxidation causes 3 main problems: it gives rise to the formation of objectionable off-flavors, it reduces the nutritional value of lipid-containing food products, and free radicals formed during oxidation may participate in the development of atherosclerosis (Taneja and Singh 2012).

Lipid oxidation can be conveniently divided into three distinct stages: initiation, propagation, and termination (Erickson 2002).

Initiation \[ \text{In}^* + \text{LH} \rightarrow \text{InH} + \text{L}^* \]

Propagation \[ \text{L}^* + \text{O}_2 (g) \rightarrow \text{LOO}^* \]
\[ \text{LOO}^* + \text{LH} \rightarrow \text{LOOH} + \text{L}^* \]
Termination

\[ \text{LOO}^•+\text{LOO}^• \rightarrow \text{LOOL}+O_2 \]

\[ \text{L}^•+\text{LOO}^• \rightarrow \text{LOOL} \]

\[ \text{L}^•+\text{L}^• \rightarrow \text{LL} \]

In the initiation step, a fatty acid radical known as the alkyl radical (L•) is formed by abstraction of a hydrogen from a fatty acid in the presence of an initiator (In•). Once the alkyl radical forms, the free radical can delocalize over the double bond system resulting in double bond shifting, and in the case of PUFAs, formation of conjugated double bonds. In bulk oils, the ease of formation of alkyl radicals in fatty acids increases with increasing unsaturation (Holman and Elmer 1947). The first step of propagation involves the addition of oxygen to the alkyl radical resulting in the formation of the peroxyl radical (LOO•), which has a higher energy than the alkyl radical. Thus, the peroxyl radical can abstract hydrogen from another unsaturated fatty acid, and produce a lipid hydroperoxide (LOOH) and a new alkyl radical. The acceleration of lipid oxidation in emulsions is the decomposition of lipid hydroperoxides (LOOH) into highly reactive peroxy, and alkoxyl radicals by transition metals. These radicals react with unsaturated lipids (LH) within the droplets or at the oil-water interface, which leads to formation of lipid radicals. The lipid oxidation progresses as these react with other lipids in vicinity (McClements and Decker 2000).

The primary lipid oxidation products are lipid hydroperoxides. They may further decompose into secondary oxidation products such as highly reactive, and cytotoxic 4-hydroxy-2-alkenals. These compounds were found to affect cell viability, even at low concentrations. They have a toxic effect on L6 muscle cell lines. There is also uncertainty in the bioavailability of protein bound 4-hydroxy-2-alkenals. Several methods have been
used to measure a variety of oxidation products. Lipid hydroperoxide is one commonly measured parameter. The iodometric titration method is one traditional method for measurement of hydroperoxides. Peroxide value (PV) was measured using spectrophotometer to evaluate the oxidative stability of the emulsions for current research (Shantha and Decker 1994). Commercial kits are also available for measurement of PV and secondary oxidation products (PV, alkenals).

The primary products including hydroperoxides break down by various means to secondary oxidation products, which are odoriferous in nature. The thiobarbituric acid reactive substances (TBARS) assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been criticized for its reactivity towards other compounds other than malonaldehyde (MDA). MDA a secondary decomposition product of PUFAs with three or more double bonds reacts with thiobarbituric acid (TBA) to form a stable pink chromophore with maximal absorbance at 532 nm. The reaction with TBA occurs by attacking of monoenoic form of MDA on the active methylene groups of TBA. Secondary oxidation products so formed may be aldehydes, ketones, alcohols or hydrocarbons (Robards and others 1988). TBARS used as a measure of secondary lipid oxidation reaction products (McDonald and Hultin 1987).

2.3 Xanthan gum

Xanthan gum is anionic polysaccharide produced by the fermentation of Xanthomonous campestris (Khouryieh and others 2007). This organism is found in nature on the leaf surfaces of green vegetables, particularly the cabbage family. It is a natural,
high molecular weight polysaccharide that provides stabilization, suspension, and thickness in the finished product. The molecular weight of xanthan ranges from $2 \times 10^6$ to $5 \times 10^7$ Da depending on the associations between chains (Long and others 2013).

The main chain of xanthan is built up of D-glucose units linked through the β-1 position of one unit with 4th position of the next unit (Fig 2.2). This linear backbone is identical to the chemical structure of cellulose. The primary structure of xanthan consists of pentasaccharide repeating units. The presently accepted structure of xanthan consists of $(1 \rightarrow 4)$-β-D-glucopyranose units. Trisaccharide side chains are attached to alternate sugar residues on the main chain at the C-3 position. The side chain consists of two mannose residues and a glucuronic acid residue (Khourieh and others 2007). The terminal β-D-mannopyranose residue is $(1 \rightarrow 4)$ linked to the β-D-glucuronic acid residue, that in turn is $(1 \rightarrow 2)$ linked to non-terminal α-D-mannopyranose residue. The 6-OH group of the non-terminal D-mannopyranose residue is present as acetic acid ester. Pyruvate acetal groups are located on the D-mannopyranosyl end groups of these side-chains. The influence of different glycosidic (or other) linkages in the backbone of any polysaccharide is an important feature in modifying polysaccharide chain conformation and its characteristics (Sanderson 1981).

Xanthan of different pyruvate levels (that is 1% to 6%) displays different rheological properties. Pyruvic acid attached to the terminal carbohydrate of the side chains adds another carboxylate group (Harding and others 1994). Acetyl and pyruvate substituents are linked in variable amounts to the side chains, depending upon which Xanthomonas campestris strain the xanthan is isolated from. The pyruvic acid content also varies with the fermentation conditions. On average, about half of the terminal
mannoses carry a pyruvate, with the number and positioning of the pyruvate and acetate residues conferring a certain irregularity to the otherwise very regular structure. Usually, the degree of substitution for pyruvate varies between 30% and 40%, whereas for acetate the degree of substitution is as high as 60-70%. Some of the repeating units may be devoid of the trisaccharide side chain (Sworn 2002; Whitcomb and others 1978).

Fig 2.2. Chemical structure of xanthan gum (Harding and others 1994).

The secondary structure of the xanthan depends on the conditions under which the molecule is characterized. The molecule may be in an ordered or disordered conformation. The ordered conformation may be of either native or denatured form. In native form the conformation is present at temperatures below the melting point of the molecule. This temperature depends on the ionic strength of the medium in which the xanthan is dissolved (Sworn 2002).

Native xanthan in the ordered conformation exists as a right hand helix with a pitch of 4.7 nm and a diameter of 1.9 nm. The native structure of xanthan in ordered
conformation consists of a double helix which is stabilized by non-covalent bonds such as, hydrogen bonds, electrostatic interactions, and steric effects. In aqueous solution this rigid double helix structure undergoes a conformational change, which is driven by changes in temperature and ionic strength, and also depends on the degree of ionization of carboxyl groups and acetyl contents. The temperature induced transition involves complete or partial separation of the double strand form. Renaturation of the denatured state may occur under favorable conditions. The conformation of the renatured form of the xanthan is that of an anti-parallel double stranded structure consisting of a single chain folded as a hairpin loop. The transition from the renatured to denatured state is reversible, whereas that from native to denatured state is irreversible (Harding and others 1994).

The molecular weight is roughly halfed when transition takes place from native conformation to disordered conformation. The molecular weight was found to be invariant when the transition takes place from disordered state to renatured state. The viscosity of the renatured xanthan is higher than that of native xanthan which indicates that single-stranded xanthan molecules associate during renaturation to form supramolecular structures (Sworn 2002).

The chemical structure of the XG molecule in aqueous solution confers three unique properties, which are excellent flow control, even at very low concentrations, long-lasting suspension of particulates, even in complex formulations, ability to withstand severe shear, heat treatment, enzymatic attack, and high salt levels. XG solutions are highly pseudoplastic. When shear stress is increased, viscosity is progressively reduced. When the shear is removed, the initial viscosity is regained almost
instantaneously (Sun and others 2007). This behavior of the XG is due to the ability of the xanthan molecules in solutions to form aggregates through hydrogen bonding and polymer entanglement. This highly ordered network of entangled, stiff molecules results in high viscosity at low shear rates, which accounts for the outstanding suspending properties of the XG solutions. These aggregates are progressively disrupted under the influence of applied shear (Sanderson 1981). This indicates the highly pseudoplastic behavior of the XG solutions. The high viscosity of XG solutions at low shear rates accounts for their ability to provide long-term stability to colloidal systems. The reduction in viscosity in response to increasing shear is important to the pouring properties of suspensions and emulsions, and to the efficacy of XG as a processing aid. When poured, mixed, pumped or sprayed, a XG solution will immediately show a dramatic fall in viscosity, therefore making it easy to process. When the shear force is removed, the viscosity reverts to its original level, the finished product reverts to its stable at rest form. The rigid structure of the matrix of xanthan molecules instantly dissociates when shear is applied. It then re-associates to the original structure when the shear is removed, suspending any particulates or oil droplets within it (Higiro and Herald 2007). XG acts as a long-lasting, highly effective stabilizer by holding particulates in solution for longer time. This ability of the XG is valuable to the manufacturer who wants to manufacture long shelf life products with no risk of separation and sedimentation (Catherine and others 1997).

XG is a polysaccharide which is widely used as emulsion stabilizer, thickener, and as a gelling agent in combination with other polysaccharides. The addition of XG in the emulsion formulation enhances emulsion stability. XG stabilizes the emulsions by
forming a weak gel-like structure in the continuous phase. This prevents the creaming of oil droplets since the gravitational lift on the droplets is less than the yield stress of the xanthan weak gel. XG is able to form only weak gel structures, and therefore it is not used as a gelling agent on its own in emulsion systems (Papalamprou and others 2005).

2.4. Locust bean gum

Galactomanans are polysaccharides consisting mainly of the monosaccharide mannose and galactose units. LBG, also known as Carob bean, is a galactomannan derived from the endosperm of the seeds of *Ceretonia siliqua*. The gum is finally achieved by milling the endosperms (Dey and others 2011). LBG is comprised of a high molecular weight polysaccharides composed of galactomannans consisting of a linear chain of (1→4)-linked β-D-mannopyranosyl units with (1→6)-linked α-D-galactopyranosyl residues as side chains (Fig 2.3). LBG has molecular weight range of 50,000 to 300,000 Da. The mannose: galactose ratio of LBG is approximately 4:1. The mannose and galactose content has been reported as 73-86% and 27-14%, respectively.

![Chemical structure of locust bean gum (LBG)](kawamura2008)

Fig 2.3. Chemical structure of locust bean gum (LBG) (Kawamura 2008).
The physicochemical properties of galactomannans are strongly influenced by the galactose content, and the distribution of the galactose units along the main chain. Longer galactose side chains produce stronger synergistic interactions with other polymers and greater functionality (Renou and others 2013). Since LBG is a neutral polymer and its viscosity and solubility are therefore little affected by pH changes within the range of 3-11. There are various properties which make LBG as a good choice in drug delivery. They are biocompatible, biosorbable, and biodegradable in nature. LBG is non-teratogenic and has non-mutagenic acceptable shelf-life and its degradation products are excreted readily (Dey and others 2011).

LBG is only soluble after heating up to 80-90 °C. A 1% solution has a viscosity ranging from 2000 to 3500 cps. LBG creates a pleasant texture with a good flavor release. LBG has a strong interaction with other hydrocolloids such as k-carrageenan, agar and xanthan. In the presence of kappa carrageenan and/or XG, LBG forms a gel. The synergy with kappa carrageenan provides noticeable advantages: it creates an elastic texture and thermo reversible gel. LBG is used as thickener, stabilizer, emulsifier, gelling agent. LBG is compatible with XG and forms gel (Kawamura 2008). LBG is used for a broad range of applications, including dairy products, fruit-based water gels, powdered products, baked goods (icings and cakes), dietary products, coffee whiteners, baby milk formulations, seasoning, sauces and soups and meats.

A stabilizer is any ingredient that can be used to enhance the stability of an emulsion. It can be classified either as an emulsifier or texture modifier depending on its mode of action. LBG is a plant polysaccharide which is widely used as emulsion stabilizer in food products, including cool drinks. LBG stabilizes the emulsions by
modifying the rheological properties of the aqueous phase between the dispersed particles. LBG shows strong interfacial activity in emulsions. Thus, it has both emulsifier plus stabilizer properties (Mikkonen 2008).

2.5. Interaction of xanthan with galactomannans

A synergistic interaction occurs between XG and galactomannans including guar gum, LBG and cassia gum (Dea and others 1977; Tako 1993; Tako and others 1984, 1989; Goycoolea and others 2005; Long and others; Khouryieh and others 2007a). Galactomannans are the hydrocolloids in which the mannose backbone is partially substituted by single-unit galactose side chains. The degree of substitution varies between the galactomannans, and this influences the extent of interaction with the XG. Galactomannans with fewer galactose side chains and more unsubstituted regions react more strongly. Thus LBG, which has a mannose to galactose ratio of around 3.5:1, reacts more strongly with xanthan than guar gum, which has a mannose to galactose ratio of slightly less than 2:1. XG interacts with the unsubstituted smooth regions of the galactomannans molecules. From the literature (Cheetham and others 1988) it is clearly evident that the less substituted galactomannans is the more it interacts with xanthan, thus understands why synergistic interactions are more pronounced for LBG (M/G ~3.5) than for guar gum (M/G ~1.5). At high concentrations of LBG, there is a synergistic increase in the viscosity of XG mixtures. In contrast when low concentrations are used, soft and elastic gels are formed with LBG (Higiro and Herald 2007).
2.6. Emulsions

2.6.1. Introduction

An emulsion is a heterogeneous preparation composed of two immiscible liquids (by convention described as oil and water), one of which is dispersed as fine droplets uniformly throughout the other (Eccleston 2002).

The types of the emulsion can be grouped as follows:

1. Macroemulsions
   i) Primary emulsions: Oil-in water (o/w) and water-in-oil (w/o).
   ii) Secondary emulsions: w/o/w or o/w/o.

2. Microemulsions: o/w, bicontinuous and w/o and

3. Nanoemulsions: o/w and w/o.

Macroemulsions are not thermodynamically stable. These emulsions are typically polydispersed, and their droplet diameter varies usually ranging from 1 to 100 µm.

Microemulsions contains droplets with radii < 25 nm (Rao and McClements 2011). Microemulsions, in spite of the similarity of their term emulsion, are absolutely different from macroemulsions in their physical and thermodynamic properties and their structure. They are thermodynamically stable, apparently homogenous dispersions of water in oil (w/o) or oil in water (o/w). These isotropic, solubilized systems can form in the presence of surfactants, sometimes the presence of a cosurfactant is also required. Microemulsions behave as a Newtonian liquid, so they have low viscosity (Zhao and others 2007).

However, recently increasing preference can be observed for microemulsion-based gels which can contain a viscosity enhancing agent. Nanoemulsions contains droplets of mean radii < 100 nm (Rao and McClements 2011). Unlike microemulsions, which are
transparent and thermodynamically stable, nanoemulsions are only kinetically stable (Al-Sabagh and others 2011). In the food industry, nanoemulsions are produced by high energy methods. These methods generate intense disruptive forces that mechanically break up the oil phase into tiny droplets that are dispersed within the aqueous phase.

Emulsions are thermodynamically unstable because of the positive free energy needed to increase the surface area between oil and water phases. For this reason emulsions tend to separate into layer of oil on top of layer of water with time (Decker and others 2005). An emulsion is said to be kinetically stable when there is no perceptible change in the size distribution of droplets, its state of aggregation, or its spatial arrangement over the time scale of observation, which may vary from hours to months depending on the material (Vega and others 2006; Dickinson 2003). Surfactants, proteins and/or thickening agents can be used to increase the kinetic stability of the emulsion. Generally, emulsion breakdown can be due to flocculation, coalescence, sedimentation, creaming, Ostwald ripening and phase inversion. The long term stability of the emulsions is normally extended by adding a variety of stabilizers. The stabilization can be sought either through control of rheology of the continuous phase, i.e. by enhancing its viscosity or through control of interfacial properties (McClements 1999).

Flocculation is the result of attractive forces between the droplets and leads to the formation of flocs (Fig 2.4) of dispersed phase. It may occur in biopolymer stabilized emulsions, either by depletion or bridging mechanisms (Blijdenstein and others 2004). Depletion flocculation arises when biopolymer concentration exceeds a critical value. The presence of non-adsorbing or excess biopolymer in the continuous phase increases the attractive force between the droplets by osmotic effect and provokes the exclusion of
the biopolymer chains from the narrow region surrounding two droplets. This depletion mechanism is not specific to biopolymers (McClements 2000).

Fig 2.4. Flocculation (McClements 1999).

Depletion flocculation can also be induced by non-adsorbed small surfactant micelles and by inorganic or organic nanoparticles. On the other hand, bridging flocculation occurs when a single biopolymer molecule adsorbs at the surface of more than one emulsion droplet. It thus acts as a polymeric link and promotes bridging flocculation (Guzey and McClements 2007). The formed flocs are the first step to droplet sedimentation (in W/O emulsions) or creaming (in O/W emulsions) and coalescence.

Coalescence is the process where two or more droplets merge together to form a single larger droplet (McClements 1999). For coalescence to occur, the droplets need to be in close proximity for a long period of time. This situation occurs in creamed or flocculated emulsions. Coalescence is an irreversible process. It involves rupture of the thin liquid film of the continuous phase that separates dispersed oil droplets in a concentrated emulsion or in a cream. If the droplets have a lower density than the surrounding liquid, they have a tendency to move upward, which is referred to as
creaming. This is essentially related to the difference in the density between the dispersed phase and the continuous phase. The rate of creaming or sedimentation can be described by Stoke’s law. In the case of a concentrated emulsion the rate of creaming is lower than predicted by Stoke’s law because of the limited movements of the droplets. Creaming provides indirect information about the extent of the droplet aggregation in an emulsion (Damodaran 1997). The more the aggregation, the larger the flocs and the faster the creaming. The rate of creaming can be decreased further by increasing the viscosity of the medium, for example by adding thickening agents such as hydrocolloids and polysaccharides to the continuous phase. However, while these macromolecules can slow creaming at high concentrations, they tend to destabilize emulsions at low concentrations (McClements 1999).

**Delivery systems**

There are a number of characteristics that an edible delivery system must have if it is going to be suitable for utilization by the food and other industries. Major kinds of delivery systems are:

**Conventional emulsions**

Conventional oil-in-water (O/W) emulsions consist of oil droplets dispersed in an aqueous continuous phase, with the oil droplets being surrounded by a thin interfacial layer consisting of emulsifier molecules (Friberg and others 2004; McClements 2005).
Multiple emulsions

Water-in-oil-in-water (W/O/W) emulsions consist of small water droplets contained within larger oil droplets that are dispersed in an aqueous continuous phase. There are two different interfacial layers in this type of emulsion: the W\textsubscript{1}-O layer surrounding the inner water droplets, and the O-W\textsubscript{2} layer surrounding the oil droplets.

Multilayer emulsions

Multilayer oil-in-water (M-O/W) emulsions consist of small oil droplets dispersed in an aqueous medium, with each oil droplet being surrounded by a nano-laminated interfacial layer, which usually consists of emulsifier and biopolymer molecules (Guzey and McClements 2007).

Solid lipid particles

Solid lipid particle (SLP) emulsions are similar to conventional emulsions consisting of emulsifier coated lipid droplets dispersed in an aqueous continuous phase.

Filled hydrogel particle

Filled hydrogel particle emulsions consist of oil droplets contained within hydrogel particles that are dispersed within an aqueous continuous. They can therefore be thought of as a type of oil-in-water-in-water (O/W\textsubscript{1}/W\textsubscript{2}) emulsion (McClements and others 2007).
2.6.2. Protein-stabilized emulsions

Proteins are widely used as emulsifiers to facilitate the formation, improve the stability, and provide specific physicochemical properties to oil-in-water emulsions. There have been a number of recent advances in the understanding of the ability of various types of proteins to provide these functional properties (McClements 2005). Many proteins are able to adsorb at the interface and facilitate droplets disruption by lowering the interfacial tension (Norde 2003). However, at equivalent interfacial concentration small synthetic surfactants are generally more effective than proteins in reducing the interfacial tension. Typically, most proteins decrease the oil-water tension by about 15-20 mN/m at saturated coverage compared to 30-40 mN/m for small synthetic surfactants. The primary structure of proteins contain hydrophilic and hydrophobic residues randomly spread all over the structure. In the tertiary folded conformation, some of these residues exist as segregated patches. At the oil-water or air-water interface, protein adsorption proceeds in three main stages: diffusion from the bulk to the vicinity of the interface, actual adsorption, and reorganization of the adsorbed protein. During adsorption, only a fraction of the protein hydrophobic groups is embedded into the lipophilic phase and most of the protein structure remains into the aqueous one.

Proteins are widely used in the food industry because of the functional properties which make them of interest for flavor encapsulation (Charve and Reineccius 2009). Proteins constitute a special class of natural polyelectrolytes, simultaneously possessing several hydrophobic groups in the structure and reveal therefore a certain interfacial activity. It is known that the extent of protein adsorption is influenced by surface hydrophobicity and charge. Once adsorbed, they unfold and rearrange their secondary
and tertiary structure to expose hydrophobic residues to the hydrophobic phase. The high concentration of protein at the surface leads to aggregation and the formation of interactions. Hence, the mechanical properties of the adsorbed layer depend on the structure of the adsorbed protein, and the strength of the interactions between them (Wilde and others 2004). Upon adsorption at the O/W interface, the hydrophobic groups are attached to the surface of the oily phase or even penetrate into it, whereas hydrophilic parts protrude into the aqueous phase and provide a thick and bulky interfacial layer (Dmitry and Reinhard 2009). The described features resemble in many details the interfacial behavior of polymeric surfactants, i.e. proteins in some sense can be considered as natural polymeric surfactants. Therefore, it is not surprising that many proteins are able to improve significantly the stability of O/W emulsions. Despite the similarity with polymers, proteins differ significantly due to their very complicated structure which determines their peculiarities in stabilizing emulsions.

Protein both at interface of emulsion droplets and in the continuous phase of oil-in-water emulsions can inhibit lipid oxidation. The ability of continuous phase proteins to inhibit oxidation was due to a combination of free radical scavenging by free sulphydryl group, and chelation of prooxidant metals (Tong 2000). For example, in the casein and casein derived peptides are able to inhibit lipid oxidation by scavenging the free radical intermediates and chelating prooxidant metals (Rival and others 2001; Diaz 2003). Few proteins are able to inhibit lipid oxidation by producing cationic surface charge that repels transition metals when pH is less than the pI of the proteins.
2.6.2. Proteins and polysaccharides

An important function of the food biopolymers is to enhance the stability of food colloids. The two main types of the food biopolymers found in emulsions are proteins and polysaccharides. Polysaccharides are good stabilizing agents because of their hydrophilicity, high molecular weight, and gelationus behavior, which leads to the formation of a macromolecular barrier by increasing the viscosity of aqueous phase and slowing coalescence between dispersed droplets (Morris 2006). The mixing of proteins with polysaccharides considerably enhances the stability of emulsions. Polysaccharides are most often employed as additives, especially in pharmaceutics and the food industry. Mixing, for example, two charged biopolymers in aqueous solution, electrostatic and/or hydrophobic interactions between the components takes place, and three different scenarios are possible: cosolubility, incompatibility, or complex coacervation (Dmitry and Reinhard 2009). The combination in the same system of the advantages of proteins (fast adsorption compared to polysaccharides) and polysaccharides (steric repulsion or viscosity enhancement) to stabilize emulsions is increasingly studied. Proteins and polysaccharides both contribute by their emulsifying/stabilizing properties to create novel emulsions with improved stability and functionality. The development of surface active protein-polysaccharide complexes can be achieved either by covalent bonding or electrostatic interactions. Covalent linkage of protein to polysaccharide can be achieved by direct chemical means (Diftis and others 2005), conjugation (Boeriu and others 2004) and dry heat induced conjugation (Oliver and others 2006). It is important to wisely choose the polymers and physicochemical conditions so that the protein and polysaccharide carry opposite charges. Another way to stabilize emulsions is to combine
protein and polysaccharide properties without developing any attractive interactions between the two biopolymers (Benichou and others 2002).

An extreme type of protein-polysaccharide interaction occurs when a covalent linkage is formed between the two biopolymers, creating a new amphiphilic biopolymer with improved surface properties. Protein-polysaccharide conjugates can be formed without using any chemicals, generally by linking protein-amino-groups. More often protein-polysaccharide complexation arises from non-covalent association, mainly driven by attractive electrostatic interactions. Numerous studies (Benichou and others 2007; Long and others 2013; Renou and others 2013) have shown improved emulsion stability, attributable to the presence of associative interfacial interactions between the protein and polysaccharide. One of the procedures for the emulsion formation is preparing a mixed solution of the biopolymers and using the resulting protein-polysaccharide complex for the emulsification.
Chapter 3- Materials and methods

3.1. Materials

Menhaden oil (14:0 myristic acid 6-9%, 16:0 palmitic acid 15-20%, 16:1 palmitoleic acid 9-14%, 18:0 stearic acid 3-4%, 18:1 oleic acid 5-12%, 18:2 linoleic acid < 3%, 18:3 linolenic acid < 3%, 20:4 arachidonic acid < 3% 18:4 octadecatetraenoic 2-4%, 20:5 eicosapentaenoic 10-15% and 22:6 docosahexaenoic 8-15%) was obtained from Sigma Aldrich, Co. (St. Louis, MO). WPI was obtained from Davisco Foods Int’l, Inc. (Le Sueur, MN). XG and LBG were purchased from Sigma Aldrich, Co. (St.Louis, MO). Iron(II) chloride tetrahydrate, xylenol orange disodium salt, 2-thiobarbituric acid, iron(III) chloride, methanol, 1-butanol, 1,1,3,3-tetraethoxypropane were purchased from Sigma Aldrich, Co. (St.Louis, MO). Hydrogen peroxide was obtained from Fischer Scientific (Fair Lawn, NJ). All other reagents were of analytical grade or purer.

Deionized water was used to prepare all the emulsions.

3.2. Emulsion preparation

WPI solutions (10%), XG and LBG (1%) were prepared separately by dissolving measured quantities of WPI, XG and LBG powders into deionized water at room temperature, followed by stirring for 6 hr to ensure complete dispersion, and the solutions were heated for 30 min at 80 °C in water bath. The O/W emulsions were prepared first by mixing required amount of deionized water to menhaden oil, then adding solution of WPI; to this XG and LBG were added. The mixed solution of WPI, menhaden oil, XG and LBG was first emulsified using a homogenizer (PowerGen 500, Fischer Scientific) for 15 min at 300 W output power. The emulsions were then sonicated in an ultrasonic
water bath (B 1500A-MT, VWR, San Francisco, CA) for 1 min at high speed. The pH of the final emulsion was 6.6. Sodium azide (1%) was added into the final emulsions as an antimicrobial agent. The final composition of the emulsion was 20% v/v menhaden oil, 2 wt% WPI, 0-0.5 wt% XG, 0-0.5 wt% LBG and 0-0.5 wt% XG/LBG mixtures. XG and LBG were blended in 50:50 synergistic ratios for the XG/LBG mixtures.

3.3. Creaming index

Creaming index provides indirect information about the extent of the droplet aggregation in an emulsion. The more the aggregation, the larger the flocs and the faster the creaming. Immediately after preparation, emulsions were transferred to 21 mm diameter, 70 mm high glass test tubes and were sealed to prevent evaporation. They were kept at ambient temperature, and the movement of any creaming boundary was tracked for period of 15 d. Emulsions separated into top cream layer and a bottom serum layer was monitored. The total emulsion height ($H_T$) and serum layer height ($H_S$) were measured. The creaming index was reported as $CI (%) = 100(H_S / H_T)$.

3.4. Microstructure

Microstructure of the emulsions was studied using a deconvolution microscopy (Zeiss Axioplan IIe imaging, Carl Zeiss microscopy LLC, Thornwood, NY) equipped with attached camera. A 10x objective magnification was used to visualize the images at 50 mm below the cover slip. The 50 µL of freshly made emulsion droplet was taken on 1.2 mm thickness of glass slide. A coverslip was placed on the top of the droplet ensuring
no air gap or bubbles were seen. The images were processed using the software axiovision.

3.5. Lipid oxidation measurements

The effect of the gums concentration on stability of WPI-stabilized emulsions at room temperature was investigated during 8-week storage time in sealed 2 mL vials. Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were measured to evaluate the oxidative stability of the emulsions.

Peroxide value

The PV was determined using the method described in detail by Shantha and Decker (1994). The PV was determined by adding 100 mg of emulsion to 9.9 mL of 7:3 chloroform/methanol solutions and the sample was vortexed for 2-4 sec. A 50 µL of 10 mM xylenol orange was added to the sample and vortexed 2-4 sec and then 50 µL iron (II) chloride was added and vortexed again. After 5 min of incubation at room temperature the absorbance of the sample was determined at 560 nm using spectrophotometer UV-1201 (Shimadzu, Kyoto, Japan). A standard curve (Fig 3.1A) of Fe$^{+3}$ vs Absorbance was constructed using a standard solution of an iron(III) chloride (10 µgFe/ml).

TBARS value

TBARS was determined using the method described by Ronald and others (2005). The TBARS was determined by dissolving 100 mg of emulsion in 25 mL of 1-
butanol. A 5 mL of sample solution was transferred to dry screw cap glass test tube. To the sample solution 5 mL of 0.2% thiobarbituric acid was added and glass tubes were vortexed. After 2 hr of incubation in a water bath at 95 °C the tubes were cooled under water for 10 min. After cooling, the absorbance was measured at 532 nm using a spectrophotometer UV-1201 (Shimadzu, Kyoto, Japan). TBA values were determined using a standard curve (Fig 3.1B) made from 1,1,3,3-tetraethoxypropane(TEP). TBARS were expressed in µmol of malonaldehyde per/g of emulsion.

3.6. Viscosity measurements

Emulsion viscosity was measured using a Brookfield viscometer DV-II + Pro (Brookfield Engineering, Middleboro, MA) using spindle-2 at a speed of 100 RPM within 24 hr of preparation.

3.7. Statistical analysis

All experiments were carried out at least in duplicate. The results were reported as the mean ± standard deviation (SD) of these measurements. Significant differences were analyzed using a two-way or one-way ANOVA procedure, as appropriate, followed by Bonferroni’s and LSD post-hoc comparisons tests. Level of significance was set at p < 0.05. Statistical analyses were conducted using the SAS (version 9.1.3) software (SAS Institute Inc., Carry, NC).
Fig 3.1. Plot of standard curve A) Peroxide value B) TBARS.
Chapter 4- Results and discussions

4.1. Viscosity measurements

Viscosity values increased with the increase in the concentration of gums in the emulsions (Fig 4.1). The apparent viscosity values in WPI-stabilized oil-in-water emulsions containing LBG alone were significantly lower (p < 0.05) than those with either XG or XG/LBG. This is because of weak polymer intermolecular interaction of neutral polysaccharide with the emulsion (Dea and others 1977). Emulsions containing XG showed improved viscosity when compared to the emulsions containing LBG. The emulsion viscosity gradually increased with the increased concentration of XG and XG/LBG mixtures from 0 to 0.15 wt% and sharply increased when concentration exceeded 0.15 wt%. These results are in agreement with Sun and others (2007) who reported sharp increase in the emulsion viscosity when XG concentration exceeded 0.2 wt%. This indicates that the viscosity of an emulsion is directly proportional to the viscosity of the continuous phase, any alteration in the rheological properties of the continuous phase has a corresponding influence on the rheology of the whole emulsion (McClements 1999). The viscosity of the emulsions containing XG/LBG mixtures was significantly higher (p < 0.05) than the emulsions containing either XG or LBG alone. The viscosity was sharply enhanced at higher concentrations of XG/LBG mixtures, which explains the synergistic interaction between anionic XG and nonionic LBG gum mixtures due to the intermolecular binding that occurs between side chains of the xanthan and locust bean gum or due to cooperative interactions between ordered segments of XG and unsubstituted zones of the LBG mannan backbone or could be interactions between disordered segments of xanthan molecules and LBG (Renou and others 2013).
Fig 4.1. Viscosity values of 2 wt% WPI stabilized emulsion with 20% v/v menhaden oil at different gum concentrations (wt%). Data represents means (n=2) ± standard deviations. Some error bars within data points. Means with same superscript are not significant. (LBG- Locust bean gum, XG-Xanthan gum, XG/LBG-Xanthan/Locust bean gum mixture).

4.2. Microstructure

Microstructure of the emulsions with LBG, XG and XG/LBG mixtures at various concentrations is shown in Fig 4.2. Emulsions containing LBG appear to be flocculated at all of the concentration levels. In the presence of ≥ 0.05 wt% XG (Fig 4.2B), oil droplets appear partially flocculated with more empty spaces between the droplets and the extent of flocculation increases slightly with increasing XG concentration up to 0.15 wt%. At 0.2 wt% concentration, oil droplets show considerable flocculation, but with smaller
flocs. In the presence of 0.5 wt%, little or no flocculation was observed. In contrast, emulsion containing a concentration of 0.5 wt% appears with little or no flocculation. This is probably due to increased viscosity of the high concentration, which immobilizes the oil droplets and prevents them from coming close together. Similar results were reported by Sun and others (2007). In the emulsions containing XG/LBG mixtures (Fig 4.2C), the flocculation was much lower at ≤ 0.1 wt% when compared to either of the gum alone. At > 0.15 wt% XG/LBG concentration, oil droplets appears to show little or no flocculation. This could be explained by the kinetic energy barriers associated with the restricted movement through highly viscous or networks of XG/LBG mixtures, which in turn influenced the rate and extent of phase separation in creaming.
Fig 4.2.
Microstructures of 2 wt% WPI stabilized emulsion containing 20% v/v menhaden
A) LBG; B)XG; C) XG/LBG: i) 0 wt% ii) 0.05 wt% iii) 0.1 wt% iv) 0.15 wt% v) 0.2 wt% vi) 0.5 wt%.
Scale-50 µm (LBG- Locust bean gum, XG-Xanthan gum, XG/LBG- Xanthan/Locust bean gum mixture).
4.3. Creaming stability

Creaming stability was investigated to evaluate the relative stability of O/W emulsions. The creaming profile of the emulsions with different gum concentrations is shown in Fig 4.3. The creaming index of the emulsions as a function of time was plotted in Fig 4.4. The visual examination of the emulsions over a period time shows slight phase boundary differentiation in few of them, and there was no separation in few. The emulsions with different concentrations of LBG showed serum separation even at high concentrations. The serum phase remained clear throughout the storage in these emulsions. The emulsion containing all LBG concentrations showed rapid cream separation for first 3 hr except 0.5 wt% and then gradually slowed after 24 hr and reached close to the plateau value for the emulsion stabilized by WPI alone (control). Around 60% creaming was observed at 24 hr for 0.05 wt% of emulsion containing LBG. Similar creaming behaviors of different concentrations of XG were explained in Sun and others (2007). The creaming was extremely rapid at 0.05 wt% of XG alone at 24 hr with the creaming of 66%. Creaming rate was ended as 24.53% and 19.7% at 24 hr for emulsions containing 0.1 wt% and 0.15 wt% of XG respectively. Addition of 0.2 wt% XG along with WPI significantly decreased the creaming of the emulsion compared with lower concentrations and serum separation was observed after the 96 hr storage. The emulsions with 0.5 wt% XG did not show any phase separation during the storage period of 15 d. This is due to sharp increase in viscosity at 0.5 wt%, which prevented the fluid from moving there by decreases creaming rate. In the presence of XG/LBG mixtures, the emulsions containing 0.05 wt% showed a small phase separation at 18 hr of storage time, whereas 0.1 wt% showed a phase separation after 168 hr. At 0.15 wt% or higher, no
distinct serum separation was observed during the 15 d storage time. There was significant (p < 0.05) difference between XG and XG/LBG emulsions for all concentrations except for 0.2 wt% and 0.5 wt%.

Depletion flocculation and thermodynamic incompatibility are the two main mechanisms which could lead to phase separation in protein-polysaccharide mixtures. In the beginning there was no clear boundary separation between the cream layer and serum layer, but as the time progress there was sharper separation due to the larger droplets moved to the top cream layer and small ones to the bottom in the emulsions containing all the concentrations of LBG and XG concentrations with < 0.5 wt%. The creaming behaviors can be explained by flocculation from Fig 4.2. From the images the flocculation was much lower in emulsions containing XG/LBG mixtures (≥ 0.1 wt%) when compared to either XG or LBG emulsions because of the enhanced viscosity, which immobilizes the oil droplets. At higher concentration of XG/LBG mixtures (≥ 0.1 wt%) and XG (≥ 0.2 wt%), lighter depletion flocculation was observed (Long and others 2013). In this case, larger droplets did not cream faster than small ones as they were formed weak gel like network throughout the system (Dickinson 2003). Because of weak polymer intermolecular interaction of neutral polysaccharide with the emulsion, the creaming index was much faster for LBG emulsions when compared to XG or XG/LBG emulsions even with high concentrations of LBG in the emulsion. Inhibition of creaming at high concentrations (≥ 0.1 wt%) of XG/LBG mixtures is probably due to the synergistic interaction between XG and LBG, which resulted in the enhanced viscosity and form a weak gel like network. The excellent physical stability in the emulsions containing polysaccharides is likely due to the ability of the polysaccharide to increase
the viscosity of the continuous phase, which decreases droplet collisions, thus decreasing flocculation and coalescence. In addition, viscosity enhancement by the polysaccharides would decrease creaming rates (McClements 2004).

Fig 4.3. Effect of gums concentration and storage time on creaming profile of 2 wt% WPI stabilized emulsions containing 20% v/v menhaden oil. A) LBG B) XG C) XG/LBG. (i) 3 hr (ii) 240 hr. (LBG- Locust bean gum, XG-Xanthan gum, XG/LBG-Xanthan/Locust bean gum mixture).
Fig 4.4. Effect of gums concentration (wt%) on the creaming index of 2 wt% WPI stabilized emulsions containing 20% v/v menhaden oil. A) LBG B) XG C) XG/LBG. (♦ - 0%, ■ -0.05%, ▲ -0.1%, × -0.15%, ● -0.2%, ● -0.5%. Data represents means (n=2) ± standard deviations. Some error bars within data points. (LBG- Locust bean gum, XG- Xanthan gum, XG/LBG-Xanthan/Locust bean gum mixture).
4.4. Lipid oxidation

Lipid hydroperoxides are primary oxidation products that have a shorter half-life than secondary oxidation products. PV of the emulsions as a function of time are shown in Fig 4.5. PV were dramatically increased in all samples containing LBG or XG alone during the 8 week storage time. The PV for emulsions containing LBG or XG decreased with the increase in the concentration, but the decrease is not so prominent (Fig 4.5A, B). However, XG was slightly more effective at inhibiting lipid oxidation than LBG. Emulsions with XG may suppress the lipid oxidation by chelation of iron between two side chains with a pyruvate residue. Several authors (Mei and others 1998; Donnelly and others 1998; Sun and others 2007; Faraji and others 2004) reported the inhibition of the lipid oxidation by chelation of iron and therefore inactivating the peroxyl radicals. Emulsions containing XG/LBG mixtures (Fig 4.5C) had significantly (p < 0.05) lower PV at higher concentrations (≥ 0.15) than in that of emulsions containing either LBG or XG alone. At higher concentrations, the XG/LBG emulsions decreased the levels of primary hydroperoxides. This is probably due to the enhanced viscosity from the synergistic interactions between XG and LBG, which resulted in the slow diffusion of oxidants to oil droplet surface area and hence the decrease in the rate of lipid oxidation. The differences in the oxidative behavior depend on the interaction of the XG with LBG and concentration. It may also depend on the surface charge of emulsion droplets, in the decreasing order of anionic, nonionic and cationic emulsifier (Yoshida and Niki 1992).

TBARS values are an indication of secondary oxidation products formed from the breakdown of oxidized PUFAs. TBARS values of WPI-stabilized emulsions in the presence of LBG, XG and XG/LBG mixtures at different concentrations are shown in Fig
4.6. The TBARS values of the emulsions containing LBG (Fig 4.6A) rapidly increased during the storage period, but the oxidation rate in the emulsion without LBG was faster than that in the emulsions containing LBG. The TBARS decreased with the increase in the concentrations for emulsions containing LBG or XG (Fig 4.6A, B). However, the decrease in the TBARS is not so noticeable for the emulsion containing LBG or XG. The emulsion containing XG was slightly more efficient in reducing lipid oxidation than LBG. The TBARS values for emulsions containing XG/LBG mixtures (Fig 4.6C) were significantly lower than in emulsions containing either XG or LBG alone. Significant differences (p < 0.05) were observed between the LBG and XG/LBG emulsions at all concentration levels. When compared with XG, emulsions containing XG/LBG mixtures had significantly (p < 0.05) lower TBARS values only at higher concentrations (≥ 0.15 wt%). At higher gum emulsion concentration, the packing of surfactant molecules at the oil-in-water interface is tighter; hence, the membrane acts an efficient barrier to the diffusion of lipid oxidation initiators into oil droplets (Coupland and others 1996). The lipid oxidation was retarded more effectively at ≥ 0.15 wt% in the XG/LBG emulsions, which shows ability to inhibit the inactive peroxyl radicals in the emulsified oil by the combination. TBARS measurements showed that the emulsions made with ≥ 0.15wt% XG/LBG mixtures offered better protection against lipid oxidation when compared to 0.5 wt% XG emulsions or against all the concentrations of emulsion containing LBG.
Fig 4.5. Effect of gum concentration (wt%) on peroxide value (PV) of 2 wt% WPI stabilized emulsions containing 20% v/v menhaden oil. A) LBG B) XG C) XG/LBG. ■ - 0%, ▲ -0.05%, x -0.1%, ●-0.15%, ● -0.2%, + -0.5%. Data represents means (n=2) ± standard deviations. Some error bars within data points. (LBG- Locust bean gum, XG-Xanthan gum, XG/LBG-Xanthan/Locust bean gum mixture).
Fig 4.6. Effect of gums concentration (wt%) on TBARS value of 2 wt% WPI stabilized emulsions containing 20% v/v menhaden oil. A) LBG B) XG C) XG/LBG. ■ -0%, ▲ -0.05%, x -0.1%, ✶ -0.15%, ● -0.2%, + -0.5%. Data represents means (n=2) ± standard deviations. Some error bars within data points. (LBG- Locust bean gum, XG-Xanthan gum, XG/LBG-Xanthan/Locust bean gum mixture).
Chapter 5- Conclusion and future work

5.1. Conclusion

The physicochemical properties and oxidative stability of the menhaden oil-in-water emulsions were strongly influenced by the biopolymer type and concentration level. The results revealed that emulsions containing XG/LBG mixtures had better creaming and oxidative stability than emulsions containing either XG or LBG alone. The XG/LBG mixtures in oil-in-water emulsions significantly increased apparent viscosity, decreased depletion flocculation, improved oxidative stability and reduced the creaming. The higher concentration of XG/LBG mixtures led to lower lipid oxidation rates compared to either XG or LBG alone. This research suggests that mixtures of XG and LBG could be used to inhibit the oxidation of omega-3 fatty acids in fish oils.

5.2. Future work

1. Study the influence of ionic strength and pH on the oxidative stability.

   The oxidative stability of biopolymer oil-in-water emulsions are influenced by the ionic strength and pH. At different ionic strength and pH results in the disordered structure in the gums, which induces the transition from ordered helical structure to disorder conformations. Therefore, emulsions are particularly sensitive to pH and ionic strength effects. They tend to flocculate at pH values close to the isoelectric point of the adsorbed proteins. So, the influence of ionic strength and pH play a major role in the interaction.

Microencapsulation is a process by which one material or mixture of materials is coated with entrapped within another material or system. Spray drying is the best encapsulation method to protect against oxidation, preventing flavor losses and extending the shelf life of the products. The challenges are to select appropriate concentration of the emulsion mixture to deliver the encapsulated products. The stability was evaluated during storage in air at different relative humidities.
REFERENCES


37. Cheetham NWH


