

RESEARCH ARTICLE

Effect of pH, Polysaccharide Concentrations on Properties of Lutein-loaded Oil Whey Protein Isolate in water based Emulsions

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Abstract

Physicochemical properties of emulsion based systems are influenced by various factors. In this study, lutein-loaded oil in water emulsions was prepared with whey protein isolate and selected anionic polysaccharides. Our results revealed that 0.2-1% (w/v) pectin and 0.1-0.7% (w/v) of Carboxymethyl cellulose to 1% (w/v) whey protein isolate significantly changed the electrical charge. At pH 3, the increase of HMP and LMP concentration to 1% (w/w) changed the zeta potential from +21.34 mV to -8.98 mV, while at pH 4 and 5, the increase of HMP and LMP concentration to 1% (w/w) shifted the zeta potential from 13.8 to -33.9 mV and -1.05 to -38.8 mV respectively. Increasing CMC concentration, led to change in zeta potential from +21.34 to -15.67 mV at pH 3, +13.8 to -40.22 mV at pH 4 and -1.056 to -42.45 mV at pH 5 respectively. The solubility study in MCT oil at different temperature and time 25°C for 12 h, 50°C for 6 h, 100 and 150°C for 3 min was done. Lutein solubility significantly increased with increasing solubilization temperature while lutein solubilization reached the maximum between 1 and 3% (w/v) and the solubility decreased afterward. Encapsulation efficiency was significantly high in WPI emulsion compared with other emulsion systems. However, the increase in pH from 3 to 5 decreased the encapsulation efficiency in all systems. Light microscope images of emulsion samples revealed that all droplets were spherical and this might be the anionic polysaccharides that uniformly got deposited onto whey protein isolate during homogenization. It was concluded that lutein emulsions based delivery system could be prepared using whey protein isolate as emulsifier and addition of anionic polysaccharides between their respective pI and pKa.

Keywords: Lutein, whey protein isolate, pectin, solubilization, carboxymethyl cellulose, emulsion.

Introduction

Increasing health awareness and a corresponding lifestyle are the main factors that influence the development of new food products. Lutein belongs to carotenoids, which are natural yellow, orange and red pigments coloring fruits, vegetables, leaves and flowers. These compounds are synthesized by plants whereas animals obtain carotenoids from their diet (Ramesh *et al.*, 2015). Recent researchers indicated that lutein was a very sensitive molecule against light, oxygen, heat and acid conditions, it must be protected against conditions that limit stability and physiological functions (Boon *et al.*, 2010). It also has poor water solubility and low availability which limit its utilization. Nano or microencapsulation technology is the process by which core materials enriched with bioactive compounds are packed within wall materials to form capsules (Sozer and Kokini, 2009). Encapsulation technology is one of the methods used to increase stability, solubility and bioavailability of lutein in different conditions. Encapsulated lutein can be used in different food as functional ingredient (Nwachukwu *et al.*, 2016).

Different studies have been carried out on encapsulation of lutein with different wall materials. Gelatin and gum arabic were used to make lutein microcapsule, low molecular weight chitosan. Protein-polysaccharide complexes are used to stabilize acidified beverages, enhance emulsion and foam stability, micro-encapsulation of ingredients, fat replacers and meat analogues. The formation of complexes is primarily influenced by pH, biopolymer concentration and protein to polysaccharide ratio. At a certain condition of pH below pI of protein a maximum complexation is obtained with specific biopolymer ratio (Jones *et al.*, 2010). Covalent complexes of protein and polysaccharide where the protein adsorbs to the interface and covalently bound polysaccharide provides stabilization against coalescence and flocculation by electrostatic repulsion and steric hindrance (Neiryńska *et al.*, 2004). Stable emulsions using electrostatic complexes of proteins and polysaccharides can be created by preparing a primary emulsion with a protein as emulsifier thus a solution of polysaccharide is added to primary emulsion (Grigoriev and Miller, 2009).

The emulsion is allowed to equilibrate for some time and adjusted to desired pH. Whey protein isolate is milk protein and consist of beta lactoglobulin (50%), bovine serum albumin, alpha lactalbumin and immunoglobulins and has its isoelectric point at pH 5 (Smithers, 2015). Whey protein isolate was used to stabilize oil in water emulsions due to its amphiphilic nature consequently at pH closer to isoelectric points. Coalescence, aggregation flocculation occurred due to a reduced steric repulsion between protein molecules (Kulmyrzaev *et al.*, 2000; Qiu *et al.*, 2015; Davidov-Pardo *et al.*, 2016). Polysaccharide such as Carboxymethyl cellulose (CMC) and pectin has been used in stabilization of oil in water (O/W) emulsions. CMC is an anionic polysaccharide obtained from cellulose. CMC readily dissolves in water to form viscous solutions with a range of thickening, stabilizing and film-forming properties (Liu *et al.*, 2008). CMC were used to stabilize O/W and the results revealed that it can increase emulsions that stabilize through increase viscosity of water phase that reduce droplets movement (Paximada *et al.*, 2016). On the other hand, pectins are composed of a mixture of methyl esterified galacturonan, galactan, and araban have a pKa between 2 to 4 and are classified as high methyl or low methyl pectin depending on the degree of esterification and their degree of esterification is greater or less than 50% (Munarin *et al.*, 2012). Pectin emulsion stability was mostly associated to droplets size distribution, viscosity and less droplets movements (Lutz *et al.*, 2009).

Different works have been done on the improvement of lutein stability and solubility using various wall materials. Qv *et al.* (2011) and Arunkumar *et al.* (2013) used molecular weight chitosan and gelatin/gum Arabic to produce lutein nano and micro particles with improved stability against light and temperature. Johanna *et al.* (2013) reported the improvement of lutein release through multilayer emulsion using laccase enzymes to induce the cross-linking of beet pectin and whey protein isolate. However, information is still needed on the interaction of different wall material during the formation of lutein emulsion. Therefore, the aim of this work was to study the interaction of whey protein isolates as emulsifier and different selected anionic polysaccharides during preparation of lutein emulsion. This study will provide new reference on the preparation of lutein particles using different wall materials.

Materials and methods

Chemicals: Whey protein isolate was obtained from Hilmar Ingredients (Hilmar, California, USA). The total solid, protein, and ash in the dry power were 95.6%, 88.7%, and 2.7%, respectively. High methyl pectin with degree of esterification of 67%, low methyl pectin degree of esterification of 35% and low viscosity Carboxymethyl cellulose were supplied by CP Kelco, Shanghai, China). Lutein (purity 90%) was supplied by Zhejiang Medicine Co., Ltd (Zhejiang, China).

MCT oil having HLB value of ~11.0 (Composition: C8: 57%, C10:40%, C6:2% and C12:<1%) was a product of Lonza Inc. (Allendale, NJ, USA). All other chemicals used were of analytical grade and obtained from Sinopharm Chemical Reagent Company (China).

Preparation of stock solutions: Whey protein isolate 0.2-1% (w/v) low or high methyl pectin 0.2-1% w/v and Carboxymethyl cellulose 0.1-0.7% (w/v) solutions were prepared separately by dispersing in deionized water thus few droplets of sodium azide (0.03% (w/v)) were added. The solutions were then stirred at 250 rpm at 25°C for 3 h and kept at 4°C overnight to allow complete hydration of the biopolymers prior to further uses (Thongkaew *et al.*, 2015).

Preparation of lutein emulsion stabilized by biopolymers: Oil phase were prepared by heating MCT at 25°C for 12 h, 50°C for 12 h, 100°C and 150°C for 2 min and addition of 1, 3, 5, 7 and 9% (w/v) mL of crystal lutein and further magnetic stirred for 2 min for total dissolution. The emulsion was prepared by mixing 10 mL of oil phase with 90 mL 1% (w/v) of whey protein isolate solution. Oil phase and protein solution were mixed at 12,000 rpm for 5 min using ultraturax T-25, IKA Works Inc., Wilmington, NC, USA to obtain first emulsion. Secondary emulsions were prepared by mixing primary emulsion with low or high methyl pectin and carboxyl methyl cellulose and ultraturax at 8,000 rpm for 3 min using ultraturax T-25 according to Beicht (2013) with slight modifications.

Zeta potential: Zeta potential was measured using a Malvern Zetasizer Nano ZS analyzer (Malvern Instruments Ltd., Malvern, UK) with a He-Ne laser at 633 nm. The measurements were performed at 25°C and 173° scattering angle. The electrical charge (zeta potential) was determined from measurements of the direction and velocity that the droplets or particles moved in the applied electric field using the Smoluchowski model. The solution was used to derive the particle size distribution by a laser particle size analyzer. The refraction index applied was 1.59 for material and 1.33 for water dispersant.

Particle size measurements: The biopolymer solution and emulsions were used to derive the particle size distribution by a laser particle size analyzer (Mastersizer 2000, Malvern Corporation, England). The uniformly dispersed solution was drop-wise added into a beaker where a churn-dasher was fixed and connected with an analyzer. Once the collected frequency of particles was above a threshold, particle volume size distribution was obtained.

Lutein encapsulation efficiency: The retention of lutein was calculated by extraction method (Matos *et al.*, 2015). Five milliliters of lutein-loaded emulsion were mixed by 15 mL hexane and vortexed for 8 min at 25°C.

Free lutein was extracted from emulsion and hexane was evaporated using a rotary evaporator. Ethanol was added to free lutein followed by centrifugation at 5,000 rpm for 15 min and filtered to determine the component retained during separation processes. A standard curve of lutein in ethanol was used to determine the amount of free lutein using UV-Vis spectrophotometer at the absorbance of 446 (UV-1600; Mapada Instruments Co., Ltd., China). This calculation was used to determine the lutein encapsulation efficiency. Lutein retention efficiency was calculated as follows:

$$\text{Lutein retention efficiency} = \frac{\text{Loaded Lutein} - \text{Free Lutein}}{\text{Loaded Lutein}} \times 100$$

Light microscope: The morphology of emulsions was observed by optical microscopy (BX51, Olympus Corporation, Japan) at a magnification of 200x.

Statistical analysis: Each experiment was repeated in triplicate under the same conditions. A one-way analysis of variance (ANOVA) was applied to estimate the statistical difference. Significant differences ($p < 0.05$) between means were determined using Tuckey's tests. Statistical analyses were evaluated with SPSS software (version 19.0, SPSS Inc., Chicago, Illinois).

Results and discussion

Effect of pH on charge density of whey protein isolate and polysaccharides in water phase: The pH variation resulted in zeta potential decrease with a highest value at pH 3 and lowest at pH 6. The point of zero charge of WPI was found to be near 5 (This is ideal according the data published where isoelectric point of WPI is close to 4.6 (Fig. 1) (Smithers, 2015). Net neutrality (or isoelectric point, pI) of the WPI was found to occur at near pH 5 (zeta potential 0 mV). All polysaccharides used for this series of experiments were anionic polysaccharide containing a carboxyl groups and being its zeta potential was negative in all pH ranges studied. Their zeta potentials were negative within the pH ranges and there was magnitude decrease in charge density between pH 2-4 and these points correlate with their respective pKa (Damianou and Kiosseoglou, 2006; Jones *et al.*, 2010). The addition of polysaccharides on whey protein isolate was done between the range pKa of polysaccharides and pI of protein due to their opposite charges.

Effect of different coating materials on coarse emulsion preparation: During this study, emulsion was prepared by combination of WPI to different concentrations of polysaccharide and 10% MCT oil to form W/O emulsion. Effect of different polysaccharide concentrations on W/O emulsion at different pHs is shown in Fig. 2.

Effect of combination of high and low methyl pectin with whey protein isolate on emulsion preparation: The interaction of high and low methyl pectin (HMP and LMP) with WPI was shown in Fig. 2.

Fig. 1. pH dependence of stock solution biopolymers.

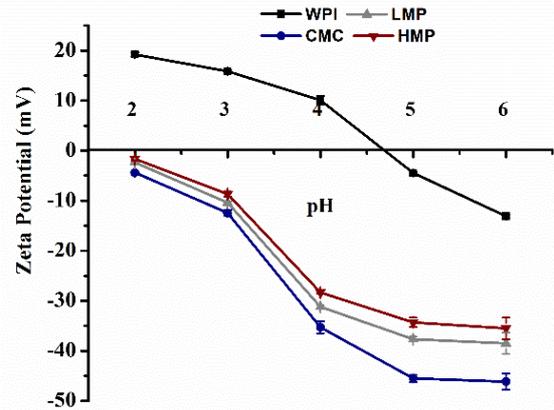
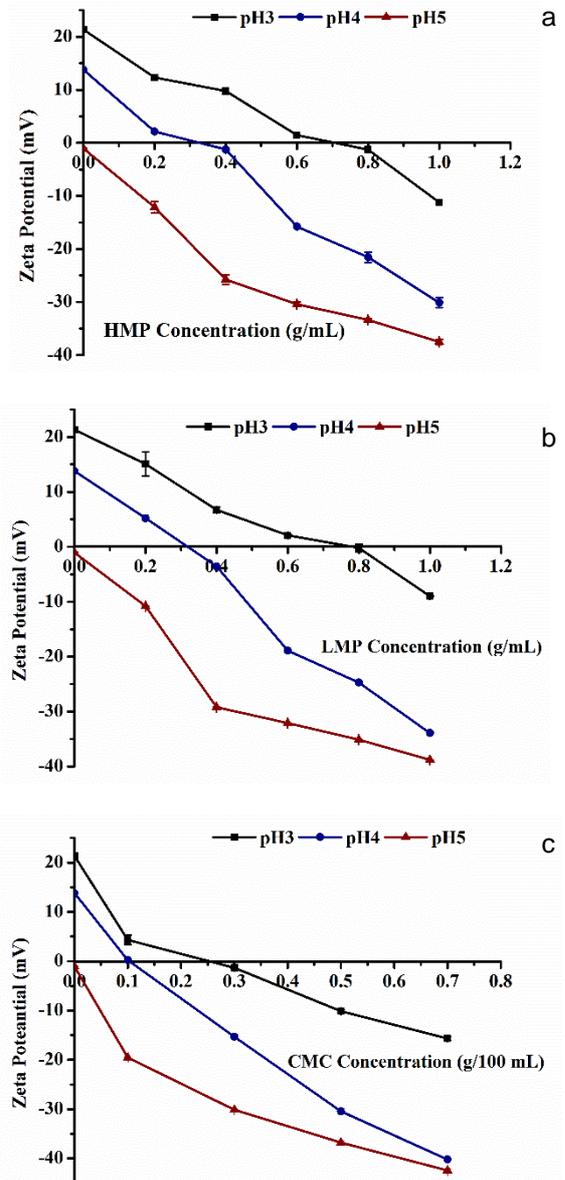


Fig. 2. Effect of polysaccharide concentration on charge density of WPI emulsions (a) HMP (b) LMP (c) CMC.



Different concentrations of HMP and LMP (0.2-1% (w/v)) were added to 1% WPI (w/w) and pH of the systems was adjusted to 3, 4 and 5. The zeta potential decreased as results of charge neutralization and some pectin could have adsorbed at positively charged protein. At pH 3, the increase of HMP and LMP concentration to 1% (w/v) changed the zeta potential from +21.34 mV to -8.98 mV. While, at pH 4 and 5, the increase of HMP and LMP concentration to 1% (w/v) shifted the zeta potential from 13.8 to -33.9 mV and -1.05 to -38.8 mV respectively. These findings suggest that pectin was adsorbed in WPI. These results are in accordance with previously reported by Patino and Pilosof (2011). It was concluded that the concentration of 1% (w/v) was the best HMP and LMP concentration for the preparation of coarse emulsion.

Effect of combination of carboxymethyl cellulose and whey protein isolate on emulsion preparation: The interaction of WPI and CMC during preparation of OW emulsion was shown in Fig. 2. Different concentrations of CMC (0.1 to 0.7% (w/v)) were added to WPI 1% (w/w) and pH of the systems was adjusted to 3, 4 and 5. Results showed that the increase of CMC concentration led to change in zeta potential from +21.34 to -15.67 mV at pH 3, +13.8 to -40.22 mV at pH 4 and -1.056 to -42.45 mV at pH 5 respectively. This charge variation might be due to CMC that adsorbed onto the droplets thus increasing the electrostatic repulsion between oil droplets. The following results are in agreement with preciously reported by Akinosho and Wicker (2015). Increasing CMC concentration over 0.5% (w/w) resulted in a more viscous solution, therefore, 0.5% (w/w) was used for further studies.

Lutein solubilization: Different lutein concentrations were solubilized in MCT at different temperatures and time (25°C for 12 h, 50°C for 6 h, 100 and 150°C for 3 min. As shown in Fig. 3, results revealed that lutein solubility significantly increased with increasing solubilization temperature while lutein solubilization reached the maximum between 1 and 3% (w/v) and the solubility decreased afterward. These results were in accordance with Heyang *et al.* (2009). Therefore, the maximum lutein solubility at 150°C and 3% (w/v) concentration was selected for further studies.

Effect of different pHs and polysaccharide on size distribution of lutein emulsion: Lutein emulsions were prepared with WPI, MCT and different polysaccharides (HMP, LMP and CMC). The effect of pH on the size distribution of the emulsions was studied as shown in Fig. 4. It is important to measure the emulsion size droplets as it is an indicator of stability. During these experiments, fresh emulsion samples were adjusted to different pHs (2-6) and size were measured. Sample with HMP, LMP and CMC had size bigger than WPI emulsions sample at different pHs. It might be due to addition of polysaccharides that led to a formation of thick layer onto droplets during homogenization.

Fig. 3. Solubility of lutein at different temperatures and time.

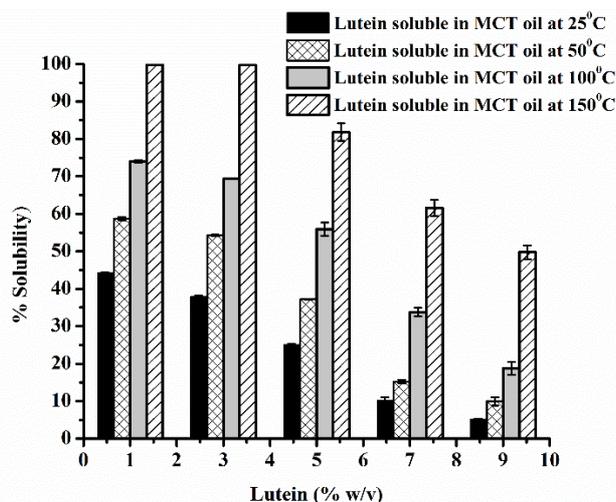
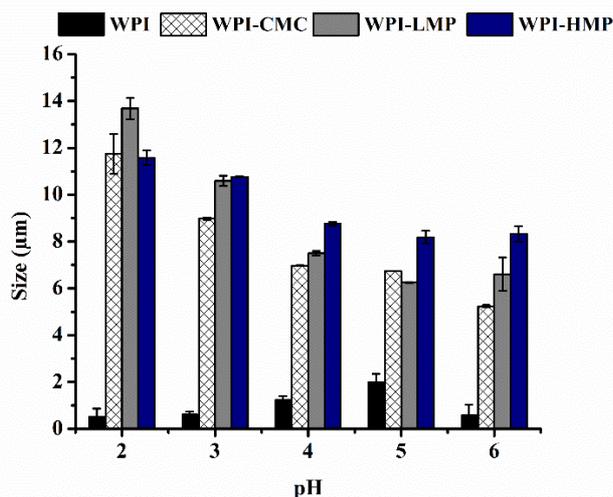


Fig. 4. pH dependence of z-average diameter fresh coarse emulsion 90 mL of 1% w/v, 100 mL of 1% w/v polysaccharides (LMP, HMP, CMC) and 10 mL of MCT with 3% w/v of lutein.



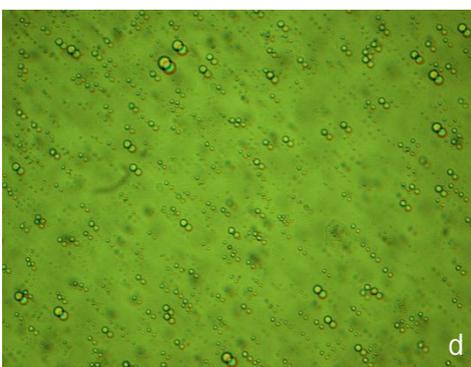
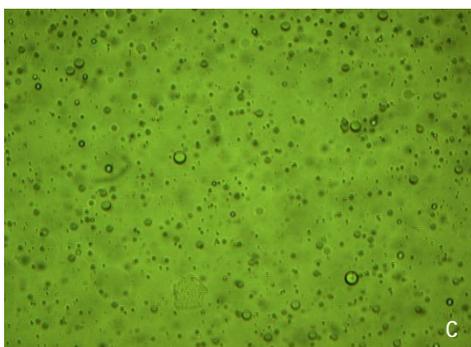
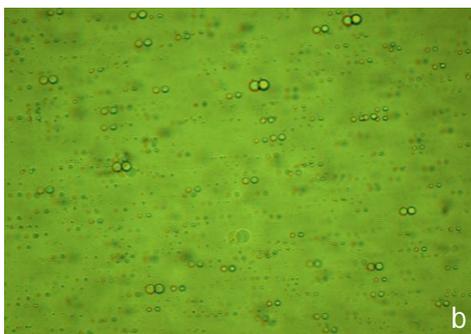
The interaction between protein and polysaccharides, dilution of protein solution might have reduced the emulsifying properties of whey protein isolate. At pH around 2, all sample had bigger size as results of complex formation between whey protein and polysaccharides. Drastic increase in size might have occurred as results of protein polysaccharides complexation below the protein isoelectric points. Similar findings were previously reported by Dickinson (2011).

Lutein encapsulation efficiency at pH between pKa and protein pl: Lutein encapsulation efficiency in WPI, WPI-LMP, WPI-HMP and WPI-CMC were evaluated at pH between pKa and pl (3, 4 and 5). As shown in Table 1, the encapsulation efficiency was significantly high in WPI emulsion compared with other emulsion systems. However, the increase in pH from 3 to 5 decreased the encapsulation efficiency in all systems.

Table 1. Encapsulation efficiency of coarse emulsion at pH 3, 4 and 5.

pH	WPI	WPI-LMP	WPI-HMP	WPI-CMC
3	96.8±0.098	95.00±0.22	91.098±0.97	77.24±0.80
4	95.77±0.271	93.22±0.13	89.456±0.57	75.693±0.35
5	73.312±0.45	83.142±0.09	78.211±1.09	66.99±0.71

Fig. 5. Images light microscope (a) WPI at pH 4 (b), WPI-LMP at pH4, (c) WPI-CMC at pH 4 and (D) WPI-HMP at pH 4.



The lutein encapsulation efficiency in WPI, WPI-LMP, WPI-HMP and WPI-CMC was reduced by 23.95%, 12.6%, 14.28% and 14.28% respectively. These results indicate that lutein encapsulation efficiency was higher around pKa (pH 3) and decreased toward protein pI (pH 5). This might be due to the addition of polysaccharides that changed the surface of the system. These results are in good agreement with the findings of Noshad *et al.* (2015).

Light microscope: Light microscope was used to look at images of fresh emulsion as shown in Fig. 5. All droplets are spherical and this might be to the anionic polysaccharides that uniformly deposited onto whey protein isolate during homogenization. These results indicated a possibility to form a stable emulsion by addition of anionic polysaccharides (HMP, LMP and CMC) to whey protein isolate loaded emulsion for lutein encapsulation and protection.

Conclusion

During this study, lutein-loaded oil in water emulsion was prepared with whey protein isolate and selected anionic polysaccharides. Emulsions preparations affected protein, polysaccharides, and MCT oil and lutein concentrations. Results also showed increasing protein concentration reduced the z average diameter and addition of polysaccharides and lutein increased the z average diameter due to adsorption onto oil surface. Light microscopes images showed more uniform and spherical oil droplets as a result of anionic polysaccharide deposition onto the surface of droplets. These findings revealed that lutein emulsions based delivery system could be prepared using whey protein isolate as emulsifier and addition anionic polysaccharides.

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