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Development of W/O/W double emulsion stabilized with milk protein concentrate-pectin complex

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Abstract

Bioactive compounds when directly incorporated into food products are prone to the processing and storage deterioration and ultimately loss of bioactivity. The present study was conducted to develop a W/O/W double emulsion to encapsulate the bioactive peptides in the inner aqueous phase (W_1), sunflower oil was used as the intermediate oil phase (O), and a combination of milk protein concentrate (MPC) and pectin was used in the outer aqueous phase (W_2) as an emulsifier and stabilizer, respectively. The emulsion was emulsified with Ultra-Turrax high shear homogenizer at 15,000 RPM for 5 min and 10,000 RPM for 3 min for the preparation of W_1/O emulsion and $W_1/O/W_2$ double emulsion, respectively. The presence of MPC along with pectin has shown to produce double emulsion with small emulsion droplets, high absolute zeta-potential, high viscosity and good physical stability in the fresh and during storage compared to double emulsion prepared only with MPC. The combined use of MPC and pectin in the outer aqueous phase is therefore proposed as a suitable food-grade strategy to enhance the structural and functional performance of peptide-loaded W/O/W emulsions.

Keywords: Double emulsion, milk protein concentrate, pectin, bioactive peptides, sunflower oil, confocal microscopy

Introduction

Water-in-oil-in-water (W/O/W) double emulsions (DEs) are colloidal delivery system (CDS) in which the small size water droplets are dispersed in larger oil droplets, which are again dispersed in a continuous external aqueous phase. The DEs are useful for encapsulating wide range of functional ingredients as a carrier delivery system as it has an additional protective layer ^[1] and has applications in food and pharmaceutical industry ^[2]. The studies conducted on DEs showed that encapsulation of bioactive compounds in inner aqueous phase have potential in preventing undesirable changes in physico-chemical properties, masks odour and flavours, bitter taste and also providing stability to encapsulated bioactive compounds and controlling the release of bioactive compounds during digestion ^[3-5]. The W/O/W DEs are preferred for encapsulating hydrophilic compounds that needs to be retained during processing and storage and also helps in targeted delivery of the bioactive compounds ^[6].

Bioactive peptides, being hydrophilic in nature can be encapsulated in W/O/W DE as they are readily soluble in water. Encapsulation of bioactive peptides in an aqueous phase of W/O/W DE can reduce their diffusion into the surrounding medium, and protecting the peptides from loss of bioactivity and other undesirable changes due to adverse conditions. The previous studies conducted of encapsulation of bioactive peptides in DE showed that the encapsulation efficiency (DE) is dependent upon the composition of the inner phase, the efficiency of the primary emulsion (W/O

emulsion), and the ability of external phase to maintain the structural integrity of the emulsion droplets ^[7].

The composition of outer aqueous phase (W_2) of DE is also important because it determines the stability of dispersed oil droplets that contains the inner water droplets. Milk proteins act as a hydrophilic emulsifier and can adsorb at the oil-water interface and forms stabilizing films. Polysaccharides present in the outer aqueous phase (W_2) increases the viscosity of continuous phase there by reducing the movement of emulsion droplets ^[9]. The combination of milk protein concentrates and pectin as polysaccharides is a promising strategy for the formation of more stable emulsion ^[10].

MPC contains casein and whey protein fractions, can be used as an emulsifier because of good surface activity and emulsifying behaviour. These milk proteins fractions can adsorb onto the newly formed oil-water interface during secondary emulsification, reducing interfacial tension and creating viscoelastic interfacial films. Pectin, an anionic polysaccharide widely used in food systems, can interact with proteins through electrostatic attraction under suitable pH conditions and can also increase the viscosity of the external aqueous phase (W_2) ^[9]. In protein-polysaccharide stabilized emulsions, pectin is often associated with improved droplet charge stabilization, reduced aggregation, and better resistance to storage-induced destabilization ^[11].

Several works for the formulation of W/O/W DE using pectin and milk proteins as an emulsifier has been conducted. The W/O/W DE stabilized with whey protein

concentrate and pectin showed improved particle size, enhanced absolute zeta potential, and better encapsulation efficiency compared with only milk protein emulsifiers devoid of pectin [9]. In another double nano-emulsion study, pectin-whey protein concentrates complexes in the outer phase produced a droplet size of 191 nm, zeta potential of -26.8 mV, and encapsulation efficiency of 91% under optimized conditions [12]. Although these studies involve whey proteins more often than MPC specifically, their mechanistic implications are directly relevant because MPC contains milk protein fractions capable of analogous interfacial interactions. The inner aqueous phase (W_1) consists of water, salt and encapsulant. Sodium chloride (NaCl) is commonly used into W_1 to increase the osmotic pressure and reduce the migration of water to the surrounding medium thereby helping to maintain emulsion droplet size [13].

This study was conducted to analyze the effects of milk protein based emulsifier and pectin on the stability of W/O/W double emulsion and their particle size analysis, zeta potential, physical stability, viscosity, encapsulation efficiency, and confocal microscopy was determined.

Materials and method

The bioactive peptide was isolated from cow milk and used as an encapsulating material in the double emulsion. MPC (~85% protein) was procured from Calpro Specialities Pvt. Ltd., New Delhi, India. Sunflower oil was purchased from local market of Karnal, Haryana. Polyglycerol polyricinoleate (Grindsted PGPR 90), hydrophobic emulsifier was obtained from DuPont-Dansico India Pvt. Ltd, India. Sodium chloride (NaCl) was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. High methoxyl (HM) pectin (GRM396) with 65.0-70.0% degree of esterification was procured from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. Other chemicals and reagents were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India.

Method

Preparation of the primary W_1/O emulsion

The W_1/O emulsion and $W_1/O/W_2$ double emulsion was prepared by modifying the procedure of [14]. For preparation of W_1/O emulsion, first aqueous phase was prepared by dissolving salt (2%) and peptide in water, simultaneously oil phase was prepared using sunflower oil and PGPR (4%) as hydrophobic emulsifier. The ratio of aqueous phase to oil phase was 30:70. The emulsion was prepared by slowly adding aqueous phase to the continuous phase under high shear mixing using an Ultra-Turrax homogenizer at 15,000 RPM for 5 min to produce very small size of emulsion droplets. After preparation of W_1/O emulsion, the $W_1/O/W_2$ double emulsion was prepared. For preparation of $W_1/O/W_2$ double emulsion, the primary W_1/O emulsion was slowly added to the outer aqueous phase (W_2) containing MPC @2% and pectin @2%, followed by secondary homogenization using Ultra-Turrax at 10,000 RPM for 3 min. The control sample was prepared using only MPC @2% in outer aqueous phase (W_2) and without pectin. The second emulsification was conducted at low shear speed compared to first emulsification, because at high speed the primary W_1/O emulsion may breakdown lead to emulsion

destabilization. The $W_1/O/W_2$ double emulsion was further subjected to different analysis for emulsion characterization.

Analysis of Emulsions

Particle size

The particle size of W/O/W DE was carried out by laser light scattering using a Malvern Mastersizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The sample was prepared by adding one part DE to 99 part double distilled water (DDW) to determine mean particle size. All the samples were analyzed in triplicate and results were expressed as micron meter (μm).

Zeta-potential

The zeta-potential of the DE was measured using Zeta-sizer (Malvern Instruments Ltd., UK). The sample was prepared by diluting DE in DDE in the ratio of 1:99. The experiment was carried out in triplicate and the results were expressed as milli-volt (mV).

Physical stability

The physical stability of DE was determined by pouring sample into glass vials and stored for 14 days at $37 \pm 1^\circ\text{C}$. The physical stability was determined by visual observation of glass vials for any phase separation on 0, 3, 7 and 14th day during storage.

Viscosity

The viscosity of DE was determined using Rheometer (Anton-Paar, MCR52) with varying shear rate (0.1 to 100 1/s) using cone and plate geometry (CP-75) attachment at controlled temperature of $25 \pm 1^\circ\text{C}$. All rheological measurements were carried out in triplicate and viscosity was expressed in mPa.s.

Confocal laser scanning microscope (CLSM)

The CLSM image analysis of W/O/W DE was carried out by staining oil phase with Nile red and aqueous phase containing MPC and pectin was stained by Fast green. The Nile red was first mixed with DE followed by mixing of Fast green in the sample. Then the sample was kept in concave microscopic slides to visualize the microstructure of DE under confocal microscope and exited at 488 nm and 633 nm for oil droplets and aqueous phase components, respectively.

Statistical Analysis

Statistical analysis of DE samples was carried out in triplicate. The mean values and standard deviations were then calculated using the statistical software (SPSS 27.0, SPSS Inc., Chicago, IL). The variance (ANOVA) of data was further analyzed using a Duncan test, in order to determine the statistical significance ($P < 0.05$) between samples. Data is presented as mean $\pm$ standard deviation.

Results and Discussion

Particle size analysis

The particle size of W/O/W DE prepared using MPC and pectin are presented in Table 1. The size of emulsion droplets was strongly dependent on the ability of the MPC-pectin mixture to stabilize the newly formed emulsion droplets during the second emulsification step. The results

showed that the presence of MPC and pectin in combination strongly affected the particle size of emulsion droplets of DE. The particle size of emulsion droplets of DE without pectin and only MPC was slightly larger compared to DE with MPC and pectin. The size of emulsion droplets is directly related to emulsion stability, as large size emulsion droplets leads to phase separation in a very quick time^[9].

These findings are in accordance with the finding of Liang et al.^[9] where small size of mean droplets were reported in the DE prepared with protein emulsifiers and pectin in combination. In another study conducted by Gharehbeglou et al.^[12], the formation of pectin-protein double nano-emulsions reached a mean droplet size of 191 nm and the presence of pectin in the outer aqueous phase stabilize the emulsion droplets due to high viscosity of continuous phase and thus maintaining the small droplets size in emulsion. In the present study, the slow growth of emulsion droplets size during storage would be the strong indicators for the proper functioning of the MPC and pectin in combination for the stabilization of double emulsion.

Zeta potential

The zeta-potential of DE was affected by the presence of the MPC and pectin as shown in Table 2. The zeta-potential of DE prepared using MPC and pectin possess higher absolute zeta-potential (-31.65 ± 1.16) compared to zeta-potential of DE prepared only with MPC (-28.37 ± 1.28). The addition of pectin to the outer aqueous phase (W₂) can alter the surface electrical properties of the emulsion droplets as pectin carries negative charges resulting in higher zeta-potential values compared to emulsion stabilized by MPC alone. The addition of pectin in combination to MPC in emulsions, significantly decreased ($P < 0.05$) the particle size and increased the surface charge potential. This may be due to the adsorption of highly negative charged complexes present in pectin on the droplet surface, which generated adequate electrostatic and steric repulsion to prevent droplets aggregation. Increased absolute zeta potential is important because it enhances droplet repulsion and lowers the likelihood of flocculation in the external aqueous phase^[15].

The high absolute zeta-potential values were reported by Gharehbeglou et al.^[12] in DE prepared using whey protein and pectin. In this study, the presence of MPC and pectin produced DE with negative zeta-potential value compared to DE prepared with MPC alone, which would have contributed to higher colloidal stability, especially when combined with the stearic protection by the hydrated pectin chains.

Physical stability

The physical stability of W/O/W DE was improved when MPC along with pectin was used compared to DE with MPC alone as shown in Fig. 1. During storage period, after 3rd day of storage period, the slight phase separation was observed in DE prepared only with MPC in the outer aqueous phase (W₂), whereas there was no separation in DE stabilized with the combination of MPC and pectin. The presence of pectin significantly improved the physical

stability of DE as it increases the viscosity of continuous phase which restricted the movement of emulsion droplets and preventing the droplet coalescence^[9]. The DE prepared only with MPC may stabilize the emulsion droplets initially at first, but over the period of time during storage, the aggregation of emulsion droplets takes place due to the low viscosity of continuous phase. Pectin, being a stabilizer increases the continuous phase viscosity and also forms thick electrostatic layer around the emulsion droplets^[9]. The study conducted on preparation of DE with whey protein concentrate and pectin showed that these complexes improved storage stability and high encapsulation efficiency compared to DE prepared only with whey protein concentrate^[9]. In the present study, the presence of pectin along with MPC serves the purpose of providing good physical stability by reducing the rate of creaming during storage and less growth in the size of emulsion droplets during storage.

Viscosity

The W/O/W DE prepared using MPC and pectin possess higher viscosity than DE with MPC alone as shown in Table 3. The addition of pectin in the outer aqueous phase (W₂) of W/O/W DE increases the viscosity of outer aqueous phase of double emulsion thereby creating resistance for the movement of emulsion droplets. Pectin when combined with adsorbed milk proteins it can create a more structured continuous phase that slows gravitational separation of droplets^[9]. This behavior is advantageous for emulsions because reduced droplet mobility lowers the chance of repeated collision and structural breakdown leading to high emulsion stability^[16].

Liang et al.^[9] studied the effect of milk protein emulsifiers along with pectin in stabilizing W/O/W DE and reported that DE stabilized with milk protein emulsifiers alone showed very low viscosity compared to DE prepared using pectin and milk protein emulsifiers which showed high viscosity and good emulsion stability. McClements (2005) reported that the viscosity of emulsion can be influenced by emulsion droplet size, phase viscosity, repulsive colloidal interactions between droplets and presence of charged droplets.

Confocal microscopy analysis

The CLSM image analysis results show the microstructure of DE stabilized with MPC and pectin in Fig. 2. The CLSM images of DEs confirmed the presence of intact small size emulsion droplets. The green background in CLSM shows the presence of milk proteins and pectin dissolved in outer aqueous phase in which the W/O emulsion are dispersed as dispersed phase as shown in red colour in CLSM image.

The CLSM images of freshly prepared samples confirmed the presence of intact double emulsion. The structural integrity of DEs weakened during storage as shown by their partial fragmentation. This indicates that the DEs matrix gets destabilized over time, leading to variations in the size and structure of DE droplets.

Table 1: Particle size of W/O/W double emulsion

Double emulsion	Particle size (μm)			
	0 th day	3 rd day	7 th day	14 th day
DE with MPC and pectin	$12.76 \pm 0.24^{\text{aA}}$	$21.34 \pm 0.53^{\text{bA}}$	$38.18 \pm 1.29^{\text{cA}}$	$65.59 \pm 2.15^{\text{dA}}$
DE with only MPC	$18.45 \pm 0.67^{\text{aB}}$	$70.86 \pm 1.43^{\text{bB}}$	$112.29 \pm 3.10^{\text{cB}}$	$167.96 \pm 5.85^{\text{dB}}$

Data are presented as average mean $\pm$ SD of three independent trials with different superscripts; a, b, c and d- superscripts differ significantly ($P<0.05$) within each row and A and B- superscripts differ significantly ($P<0.05$) within each column

Table 2: Zeta-potential of W/O/W double emulsion

Double emulsion	Zeta-potential (mV)			
	0 th day	3 rd day	7 th day	14 th day
DE with MPC and pectin	$-31.65 \pm 1.16^{\text{aA}}$	$-27.42 \pm 1.36^{\text{bA}}$	$-23 \pm 0.93^{\text{cA}}$	$-16.45 \pm 0.81^{\text{dA}}$
DE with only MPC	$-28.37 \pm 1.28^{\text{aB}}$	$-21.30 \pm 1.12^{\text{bB}}$	$-16.27 \pm 0.85^{\text{cB}}$	$-12.67 \pm 0.53^{\text{dB}}$

Data are presented as average mean $\pm$ SD of three independent trials with different superscripts; a, b, c and d- superscripts differ significantly ($P<0.05$) within each row and A and B- superscripts differ significantly ($P<0.05$) within each column

Table 3: Viscosity of W/O/W double emulsion

Double emulsion	Viscosity (mPa.s)			
	0 th day	3 rd day	7 th day	14 th day
DE with MPC and pectin	$6.51 \pm 0.25^{\text{aA}}$	$3.64 \pm 0.34^{\text{bA}}$	$0.85 \pm 0.12^{\text{cA}}$	$0.18 \pm 0.07^{\text{dA}}$
DE with only MPC	$4.73 \pm 0.86^{\text{aB}}$	$1.50 \pm 0.28^{\text{bB}}$	$0.15 \pm 0.06^{\text{cB}}$	$0.10 \pm 0.02^{\text{dB}}$

Data are presented as average mean $\pm$ SD of three independent trials with different superscripts; a, b, c and d- superscripts differ significantly ($P<0.05$) within each row and A and B- superscripts differ significantly ($P<0.05$) within each column

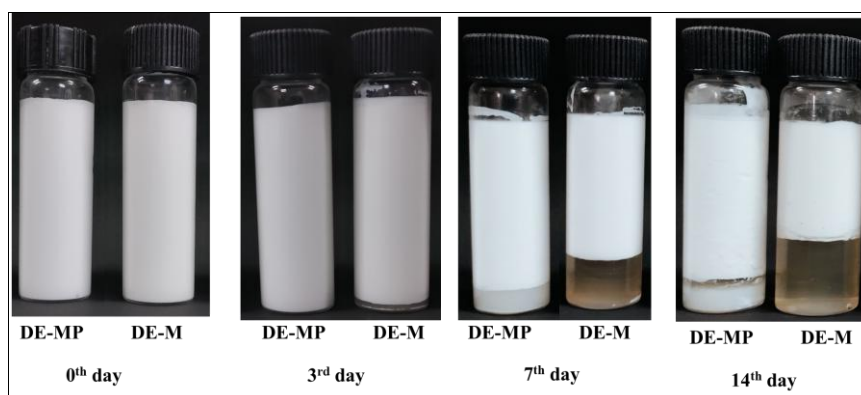


Fig. 1: Physical stability of W/O/W double emulsion during storage (DE-MP: Double emulsion with MPC and pectin, DE-M: Double emulsion with MPC)

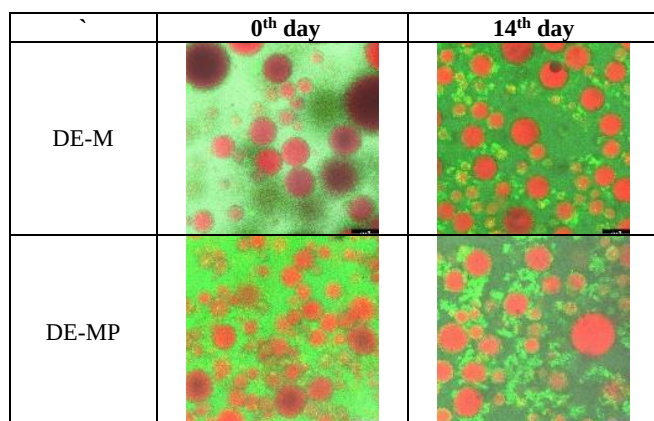


Fig. 2: CLSM analysis of double emulsions ($W_1/O/W_2$) during storage (Scale- 20 μm) (DE-MP: Double emulsion with MPC and pectin, DE-M: Double emulsion with MPC)

Conclusion

The present study demonstrated the development of a stable W/O/W double emulsion for the encapsulation of bioactive peptides using MPC in combination with pectin in the outer

aqueous phase (W_2), with sunflower oil as the intermediate phase (O) and PGPR as the hydrophobic emulsifier in the primary W_1/O emulsion. The combined use of MPC and pectin produced DE with smaller particle size, higher absolute zeta-potential, greater viscosity, and good physical stability throughout the storage period of 14 days compared to double emulsion prepared only with MPC, owing to the combined effect of protein-polysaccharide interaction at the droplet interface, which enhanced electrostatic and steric repulsion while increasing continuous phase viscosity to restrict droplet mobility and coalescence. These findings suggest the MPC-pectin complex as an effective emulsifier-stabilizer combination for W/O/W double emulsions, offering a viable and scalable strategy for the protection and controlled delivery of hydrophilic bioactive peptides in functional food applications.

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