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Effect of Combination of Tween-80 with Amphiphilic Peptides on Physical Stability of Nanoemulsion Using Low-Energy Emulsification Technique

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The current study was investigated to replace Tween-80 with peptides in order to produce physical and microstructure stability of nanoemulsion. Oil phase (2%), Tween-80 (4%) and remaining aqueous phase were taken to form nanoemulsions. In combinations of Tween-80 and peptides three ratios (1:2, 2:1 and 1:1) were taken keeping the overall concentration 4% fixed. The particle size of nanoemulsion stabilized with Tween-80, peptides and combination of Tween-80-peptides (1:2, 1:1, 2:1) were 148.71 ± 1.48, 283.66 ± 10.17, 254.50 ± 14.77, 161.90 ± 3.67, 182.80 ± 5.37 nm, showing smallest particle size with 50% replacement of Tween-80 with peptides. The zeta potential data showed competitive adsorption between Tween-80 and peptides. The polydispersity index of Tween-80:peptides (1:1) had found to be 0.33 ± 0.01 where as Tween-80 and peptides stabilized nanoemulsion had 0.20 ± 0.01 and 0.30 ± 0.05. The changes in electrical conductivity occurred maximum in case of peptides and electrical conductivity decreased with increase in concentration of Tween-80 due to preventing mobility of ions. The storage stability of nanoemulsions were found better at 4 ºC, however Tween-80:peptides (1:1) and peptides stabilized nanoemulsion had particle size growth onwards 14 days at 37 ºC. The transmission electron microscopy (TEM) image of nanoemulsion Tween-80:peptides (1:1) showed uniform distribution of peptides in combination compared with individual peptides stabilized nanoemulsion.

Keywords: Particle size, Micro structure, Phase inversion, Peptides, Emulsifier combination.

INTRODUCTION

Oil-in-water emulsions have been developed to use in many food products due to its optical transparency pertaining to small size. Emulsions are thermodynamically unstable systems that disintegrate *via* a number of processes, such as flocculation, coalescence and phase separation [1]. The formation and stability of emulsions can be improved by use of suitable emulsifiers since it adheres to the oil droplet surfaces owing to reduce the interfacial tension and enables to produce smaller particle size. Low energy techniques, such as emulsion phase inversion, phase inversion temperature and phase inversion composition, are more effective than high energy techniques because they take less energy to produce the nanoemulsion. There is transformation from a water-in-oil (W/O) emulsion to (O/W/O) at intermediate stage and finally to an oil-in-water (O/W) emulsion by under continuous mixing speed [2].

Nanoemulsions are frequently formed and stabilized using proteins as natural emulsifiers because of their high surface activity and significant protective characteristics. Synthetic surfactants like Tweens and Spans are some of the most widely used emulsifiers in commercial food and beverage products having liable to functional properties [3]. Surfactants, phospholipids and proteins, for example, are a few surface-active ingredients found in many food emulsions that compete for the oil-water interface and alter the makeup of lipid droplet surfaces. However, there is a lot of interest in using natural emulsifiers in order to produce foods that are healthier and more sustainable [4,5]. Whey proteins are considered as significant food emulsifiers due to their amphiphilic nature [6]. The protein hydrolyzates typically cover a wider region of the oil/water interface than the original protein [7]. Peptides are also less allergic, simple to digest and absorb and have a high nutritional content. These qualities are essential for parent nutrition, baby formula and sports nutrition diets [8].

In response to rising customer demand for healthier, more natural and ecologically friendly food products, the food industry is attempting to replace synthetic emulsifiers with natural ones.

The effects of combination of whey protein and Tween-80 throughout the emulsion manufacturing process to create emulsions with good physical stability [9]. The combination of sodium caseinate and Tween-20 were mixed to generate nanoemulsions based on lemon oil and mixture of emulsifiers had lower droplet sizes in comparison to nanoemulsions made with a single surfactant and remained stable for 15 days at room temperature [10]. Using cod bone hydrolyzates and Tween-20, a bilayer nano-emulsion was created and the impact of environmental stressors (such as pH, temperature and salt concentration) and long-term storage on emulsion stability was investigated [11]. The combination of Tween-80 with pea protein for effective delivery of vitamin D [12] was attempted using low-energy emulsification techniques. In present study, peptides sequence were synthesized based on the study of Saito *et al*. [13] for producing nanoemulsion individually and combination with Tween-80 using low energy emulsification technique. The purpose of current study was to understand the mechanism of action at the emulsion interface and the application potential of synthetic peptides in the formation of stable nanoemulsion that would provide a foundational investigation on the embedding and delivery of active substances using natural source of peptides as well.

EXPERIMENTAL

Medium chain triglyceride (MCT) oil was provided by Kamayani oil industries Pvt. Ltd., Mumbai, India. Tween-80 was purchased from Sigma-Aldrich Co., USA. Centrifugal membrane filters (3kDa Cutoff) were purchased from Merck Co., Darmstadt, Germany. Peptide sequence was procured from Syn-peptide Co. Ltd., China. Milli-Q water (Direct-Q®3 UV Water Purification System, Merck Life Science Pvt. Ltd., India) was used to prepare reagents required for the present study.

Preparations of peptide as emulsifier: A 0.1% peptide sample was solubilized in phosphate buffer and kept at -20 ºC until its use.

Peptide sequence:

-Leu-Glu-Glu-Leu-Leu-Glu-Glu-Leu-
Leu-Glu-Glu-Leu-Leu-GIu-Glu-Leu-

Preparation of nanoemulsions: Nanoemulsions were prepared using medium chain triglycerides (MCT) oil as oil phase and Tween-80 and amphiphilic peptides (AP). In the combinations of emulsifiers, the mass ratio of Tween-80:AP in different combinations 1:2, 1:1 and 2:1 and indicated as T1 AP2, T1 AP1 and T2 AP1, respectively. Aqueous phase was slowly added followed by gentle mixing by magnetic stirrer (IKA, China) at a speed of 1500 rpm. The concentrations of MCT oil were taken 2.0 wt.% and surfactant-oil ratio Tween-80:MCT oil was 2. The amount of water was added slowly, until a phase inversion occurred from W/O to O/W emulsion under the continuous stirring condition for 5 h [14].

Electrical conductivity: It measures the phase inversion from water-in-oil (W/O) to oil-in-water (O/W) with change in electrical conductivity during the addition of water in the oil-surfactant mixture. The MCT oil and surfactant combinations Tween-80, peptides, Tween-80:amphiphilic peptide (AP) in different combinations 1:2, 1:1 and 2:1 and indicated as T1 AP2, T1 AP1 and T2 AP1 were mixed gently. Water was added slowly to oil-surfactant mixture at the mixing speed of 1500 rpm [15]. The electrical conductivity measurement was done using a conductivity meter (Accumet AP85; Fisher Scientific, USA).

Particle size, zeta potential and polydispersity index: The mean particle diameters of nanoemulsion samples were analyzed using dynamic light scattering method (Zetasizer Nano-ZS 90, Malvern Instruments, U.K.). The samples were kept in cuvettes (10 mm diameters) and scattering angle of 90º was used to carry out the analysis. All individual measurement was an average of 3 scans. The refractive index used for the MCT oil and aqueous phases were 1.53 and 1.33, respectively. All the measurements were carried out at 25 ºC. The samples were properly diluted with water (1:100) to prevent multiple scattering.

$$D\,[4,3] = \frac{\Sigma D_i^4 V_i}{\Sigma D_i^3 V_i} \quad (2)$$

Polydispersity index (PDI) of the droplets in the emulsions was obtained by the following formula:

$$PDI = \frac{d_{90} - d_{10}}{d_{50}} \quad (3)$$

where d_{10}, d_{50} and d_{90} represent particle diameters at 10, 50 and 90% cumulative volume, respectively. The $d_{90} - d_{10}$ term indicates the width of the particle size distribution, while d_{50} shows median particle size.

Storage stability: Nanoemulsions were stored for 30 days at two different temperatures *viz*. 4 and 37 ºC. During storage the samples were drawn after every 7 days to study their storage stability.

TEM analysis: TEM micrographs were taken to the nanoemulsions prepared from Tween-80, AP and Tween-80:AP (1:1). A drop of nanoemulsion was placed on copper microscope grid coated with carbon film and then it was allowed to dry in open air. The negative staining of dried sample was done with phosphotungstic acid (2%) solution for 2 min. The microscope grid was kept for drying for 1 h before taken for analysis. The dried and stained microscopic grid containing samples were brought to analyze by the instrument.

Statistical analysis: All measurements were performed in triplicates and results were reported as mean ± standard error. Statistical analysis was carried out by analysis of variance (ANOVA) and Duncan test with confidence interval of 95% using SPSS 16 software.

RESULTS AND DISCUSSION

Particle size: The particle size obtained with Tween-80, peptides and combination of Tween-80 and peptides are shown in Table-1. In present study, the initial droplet size of nanoemulsion emulsified with amphiphilic peptide (AP) without Tween-80 was larger 283.66 ± 10.17 than that of the other samples ($p < 0.05$). Inter-estingly, the droplet size of nanoemulsion was further reduced by using a combination of Tween-80 and AP and the obtained droplet sizes of T1AP1, T2AP1

TABLE-1
PARTICLE SIZE, ZETA POTENTIAL AND PDI OF NANOEMULSIONS PREPARED USING TWEEN-80 AND PEPTIDE COMBINATION

Treatments	Particle size (nm)	Zeta potential (mV)	PDI
Tween-80	$148.71^{a} \pm 1.48$	$-7.92^{c} \pm 0.66$	$0.20^{ab} \pm 0.01$
AP	$283.66^{e} \pm 10.17$	$-10.12^{a} \pm 0.89$	$0.30^{ab} \pm 0.05$
Tween-80 + AP - 1:2 (T1AP2)	$254.50^{d} \pm 14.77$	$-7.21^{c} \pm 0.19$	$0.50^{d} \pm 0.10$
Tween-80 + AP - 1:1 (T1AP1)	$161.90^{b} \pm 3.67$	$-8.12^{b} \pm 1.51$	$0.33^{ab} \pm 0.01$
Tween-80 + AP - 2:1 (T2AP1)	$182.80^{c} \pm 5.37$	$-8.79^{b} \pm 1.81$	$0.44^{c} \pm 0.07$

Note: All the values are expressed as Mean ± SE (n = 3); Mean values with different superscripts in each column are significantly different with each other ($p < 0.05$); AP = amphiphilic peptide.

and T1AP2 were 161.90 ± 3.67, 182.80 ± 5.37 and 254.50 ± 14.77 nm, respectively. The difference is attributable to the rapid diffusion rate of Tween-80 compared to AP [16]. As a result, Tween-80 molecules are quickly adsorbed onto the oil droplet surface, producing smaller size of the droplet. This might be as a result of Tween-80 and AP having different hydrophilic lipophilic balances (HLB). In comparison to an emulsifier with a low HLB value, an emulsifier with a high HLB value is better able to stabilize an oil-in-water emulsion by creating smaller droplets [17]. Due to variations in the emulsifiers' interfacial characteristics, the inclusion of Tween-80 encouraged a reduction in particle size [4]. Proteins create a solid, viscoelastic interfacial coating that is more durable and less effective at producing droplets with smaller diameters. Additionally, the ampiphillic peptide has a slower rate of surface adsorption than Tween-80, which increases the possibility of droplet re-coalescence during the homogenization process [18]. Tween-80 has better surface activity property compared to peptides and forms a fluid adsorbed layer around the droplet. Due to its fluidity, Tween80 can move quickly from places with higher concentrations to those with lower concentrations, produces small particle size [19].

Zeta potential: High energy barriers between oil droplets are made possible by a higher absolute value of zeta potential, which results in strong electrostatic repulsion [20]. The zeta-potential value of AP and Tween-80 was -10.12 ± 0.89 and -7.92 ± 0.66 mV, respectively. The zeta-potential values of nanoemulsion combinations T1AP1, T2AP1 and T1AP2 were obtained as -8.12 ± 1.51, -8.79 ± 1.81 and -7.21 ± 0.19 respectively. As was previously indicated, peptide sequence operates as an amphiphilic surfactant, stabilizing oil droplets by electrostatic repulsion, as opposed to Tween-80, a non-ionic surfactant, which stabilizes oil droplets through steric repulsion [21,22]. This phenomenon could be explained by the law of zeta-potential being changed by the adsorption of peptides on the surface of emulsion droplets. The potential became gradually less negative as Tween-80 was added in increasing volumes to the emulsions, showing competitive displacement of proteins by the surfactant.

Polydispersity index (PDI): PDI value < 0.3 is stable and has a homogenous droplet size distribution [23]. In present study, the PDI values of Tween-80, AP and T1AP1 samples were ~0.3 or less, which suggested homogeneity and good stability of the dispersions. The PDI values of the nanoemulsion emulsified with (T2AP1 and T1AP2) 0.44 ± 0.07 and 0.50 ± 0.10, respectively increased indicating an unstable emulsion system (Fig. 1). The presence of excess peptides in the system, which was

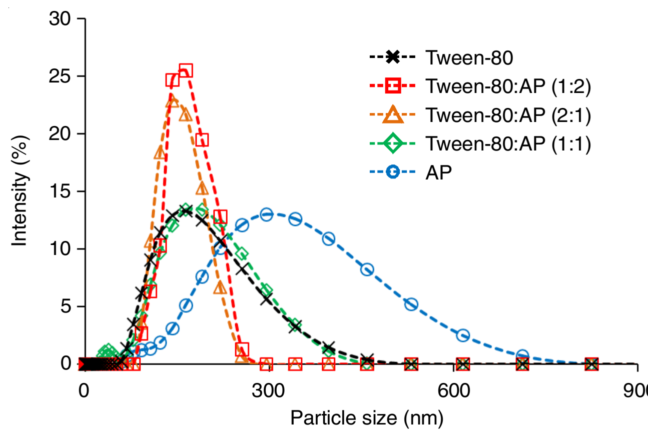

Fig. 1. Particle size distribution of nanoemulsion prepared with Tween-80 and Bovine serum albumin derived peptide (BSAP) combination: [Tween-80/; Tween-80:AP (1:2)/; Tween-80:AP (2:1)/; Tween-80:AP (1:1)/; AP/]

followed by the formation of free emulsifier micelles, is responsible for the wider PDI. These micelles may transfer oil molecules between droplets *via* an Ostwald ripening mechanism or may encourage droplet aggregation *via* a depletion mechanism. The PDI value narrowed as the emulsifier content rose until it reached a plateau [24]. Further emulsifier addition might then result in a wider PDI and consistent droplet size since the excess emulsifier caused the production of micelles.

Phase inversion: In emulsion phase inversion (EPI) method there exists change in phase from water-in-oil (W/O) emulsion, to a multiple emulsion (O/W/O) and finally to an oil-in-water (O/W) emulsion [22]. Fig. 2 represents the phase changes in nanoemulsion were measured with change in electrical conductivity on addition of water. There was less value of electrical conductivity change in case of Tween-80 whereas AP had the maximum change in electrical conductivity. Among combinations Tween-80:AP (1:2) had maximum change in electrical conductivity followed by Tween-80:AP (1:1) and Tween-80: AP (2:1). The change in electrical conductivity depend on surfactant to oil ratio (SOR) and the electrical conductivity first increase was 20%, 10%, 10% and 10% water content for SOR = 0.1, 0.2, 0.5 and 1. In case of emulsifier combinations, upon increasing the Tween-80 concentration the electrical conductivity decreased due to protective effect of Tween-80 and it was enough to prevent the interactions of ions present and thus reduction in mobility [25].

Storage study: The storage stability of nanoemulsions formed by the combination Tween-80: AP (1:1) was evaluated

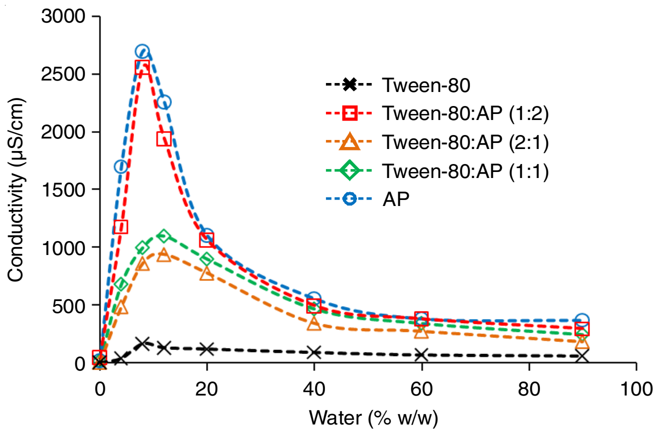

Fig. 2. Changes in electrical conductivity of nanoemulsion samples [obtained by the combinations of Tween-80 with peptides] during phase inversion process

since it produces the least particle size in above studies. The physical stability of Tween-80 and T1AP1 nanoemulsions was demonstrated at weekly intervals at 4 and 37 ºC for up to 4 weeks. The mean particle diameter did not change when stored at 4 ºC for more than 4 weeks (Fig. 3). For two weeks storage duration T1AP1emulsions remained stable and defied increases in particle diameter, but afterwards demonstrated a notable rise in diameter kept at 37 ºC. With a modest increase in thermal energy and an accompanying decrease in interfacial tension, diffusion and coalescence of droplets are likely to be the causes of consider-

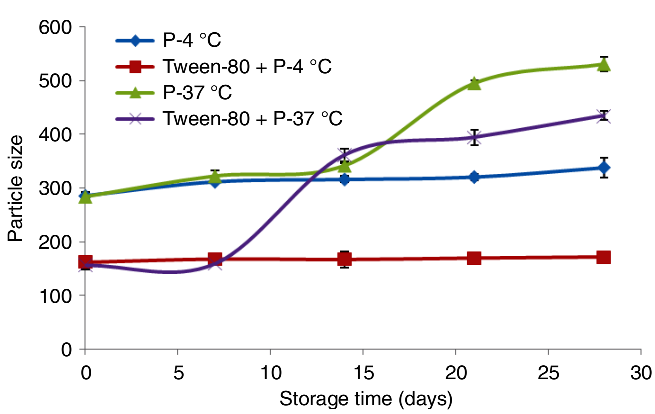

Fig. 3. Storage stability of nanoemulsion formed by the combination Tween-80: AP (1:1) with respect to peptides at 4 and 37 °C

able increase in droplet size at 37 ºC [26]. Typically, nanoemulsions degrade while being stored due to a variety of factors like gravity separation, flocculation or coalescence. In fact, emulsions stabilized with Tween-20 and potato protein hydrolyzates combination were surprisingly stable and remain homogenous after 14 days of extended storage at ambient temperature. Since less free energy is required to separate the oil phase from the water phase than is required for emulsification, nanoemulsions are thermodynamically unstable.

Microstructure: TEM image (Fig. 4) demonstrates that the nanoemulsions' surfaces were uniformly smooth and spherical. Furthermore, there was no aggregation or fusion occurring in these droplet distributions, which were uniformly scattered. The dark region in the middle of the oil droplets was observed when Tween-801:AP1 was combined, indicating a tougher or more intact membrane due to peptides. However, peptide stabilized nanoemulsions were not consistently dark, indicating that the distribution of PPH as co-emulsifier at the interface was not uniform. The study of coexistence of Tween-20 and potato protein hydrolyzates was studied by Cheng *et al.* [27] at the interface to stabilize the emulsion.

Conclusion

The replacement of Tween-80 by amphiphillic peptide (AP) has a significant effect on the physical characteristics of nanoemulsions. The use of Tween-80-AP - 1:1 (T1AP1) has better particle characteristics, polydispersity index than other combinations. In contrast, only nanoemulsions with peptides with a particle size of 283.66 ± 10.17 nm indicating that they are less effective in obtaining smaller particle size. The storage stability of nanoemulsions were found prominent at 37 ºC in peptides alone with respect to combination of emulsifiers. The microstructure of nanoemulsions matched with the particle size measurement by DLS. Thus, it can be concluded that the replacement of Tween-80 by peptide as emulsifier can provide an effective emulsifying combination having excellent physically stability that may be advantageous in the application of industrial production of food products and can be incorporated into various products.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

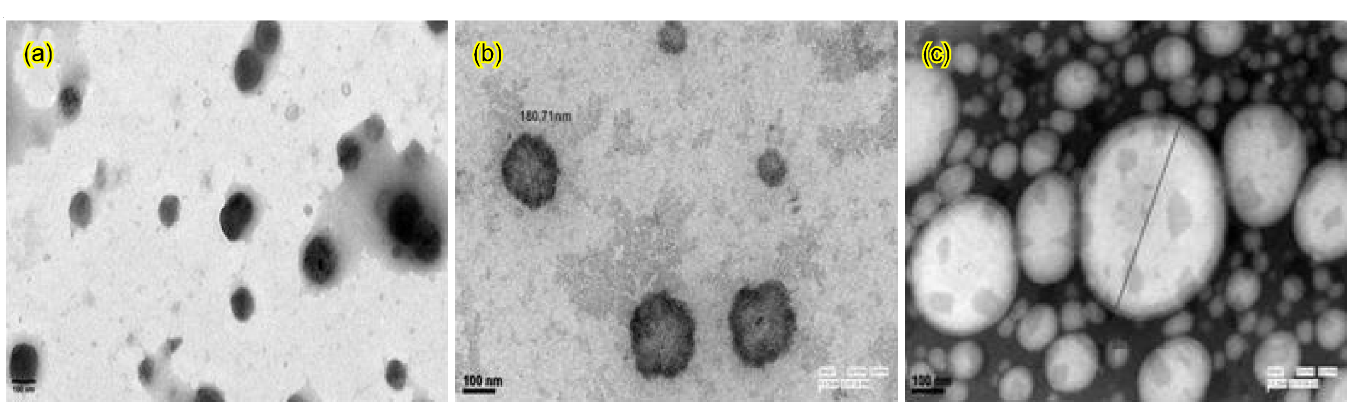

Fig. 4. TEM micrograph of nanoemulsions prepared by (a) Tween-80:AP (1:1), (b) AP, (c) Tween-80

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