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Research Article

Development and characterization of water-in-water emulsion using pea protein and different gums

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Abstract
Water-in-water (W/W) emulsions are gaining attention for their potential applications in food and nutrition due to their unique properties, including biocompatibility and stability. However, achieving stable W/W emulsions remains challenging, requiring a careful combination of biopolymers. The present study aimed to formulate W/W emulsion using a combination of protein and polysaccharide phases. Pea protein (P) was considered for the protein phase, and locust bean gum (LBG), guar gum (GG), xanthan gum (XG) were considered for the polysaccharide phase. The protein-polysaccharide phases were mixed in 10:90, 20:80, 80:20, and 90:10 ratios to create nine W/W emulsion combinations for Pea protein-Locust bean gum (PL), Pea protein-Guar gum (PG), and Pea protein-Xanthan gum (PX) each. These 27 emulsion combinations were then characterized based on their particle size, interfacial tension, phase separation, microstructure, and rheological properties. PL-6 (60:40 ratio) exhibited the smallest particle size (0.1891 ± 0.0113 µm), lowest interfacial tensions (1.78 ± 0.071 mN/m), and superior rheological properties. The emulsion phase separation study showed that the process followed the Exponential decay model, with PL-6 having the lowest decay rate constants ($k = 17.640\ s^{-1}$). The microstructure of the emulsions was revealed through Confocal laser scanning microscopy analysis. The results concluded that PL-6 emulsion proved highly effective for formulating a stable W/W emulsion. This research opens new possibilities for using such emulsions in various applications, particularly in food and nutritional security.

Keywords: Biopolymers, Pea protein, Polysaccharide, Protein, Water-in-water emulsion

INTRODUCTION

Emulsions play a crucial role in developing products with desirable textures, flavors, and nutritional profiles in food and its allied industries. Traditional emulsions, such as oil-in-water (O/W) and water-in-oil (W/O), have been extensively used due to their ability to encapsulate bioactive compounds and improve the sensory properties of food products. These emulsions are stabilized by surfactants or emulsifiers, which help maintain their stability and prevent phase separation (Espinoza-Leandro *et al*., 2023). However, they face challenges related to oxidative stability and the need for synthetic emulsifiers, which are increasingly being replaced by natural alternatives to meet consumer demand for clean-label products (Ghelichi *et al*., 2023).

Water-in-water (W/W) emulsions can be a promising solution to meet consumer demand for clean-label products and have sustainable processing technology. W/W emulsions represent a unique class of colloidal dispersions where both the dispersed and continuous phases are made of the aqueous medium. Unlike traditional O/W or W/O emulsions, these systems involve two immiscible aqueous phases, typically formed by mixing hydrophilic macromolecules that are thermodynamically incompatible (Esquena, 2016). These emulsions are characterized by ultralow interfacial tensions and biocompatibility, making them suitable for applications where traditional emulsions might not be ideal (Wang *et al*., 2022).

The ability of W/W emulsions to preserve the stability of bioactive substances without requiring organic solvents has drawn attention to their potential applications in diverse fields such as food and nutrition, cosmetics, and pharmacy (Chen, Guo, *et al*., 2021a; Hann *et al*., 2017; Tea *et al*., 2020, 2021; Yang *et al*., 2020). It is particularly advantageous for the encapsulation and controlled release of nutrients and bioactive compounds, which can be beneficial for creating functional foods with enhanced nutritional profiles (Kadi *et al*., 2022; Muschiolik & Dickinson, 2017). W/W emulsions can be used to create structured food materials, such as gelled emulsions, which serve as fat replacers in meat products (Aledo, 2015). W/W emulsion formulated from xanthan gum and whey protein isolate was identified for its encapsulation and protection of riboflavin, from degradation due to environmental factors such as light and pH changes (Pu *et al*., 2024).

Even though W/W emulsion stabilization is a challenging issue, the pickering effect by biopolymer microgels (Jordán, 2018), innovative techniques like microchannel emulsification and ultrasonic treatment and incorporation of natural stabilizers such as plant-derived polysaccharides and protein (Balcão *et al*., 2015; Schubert *et al*., 2006) are few methods to address the challenges with simultaneous improvement in emulsion properties.

Proteins and Polysaccharides are vital nutrients in the human diet, and their deficiency can lead to serious health conditions such as kwashiorkor and marasmus (Arcieri *et al*., 2021). Currently, the importance of plant protein is increasing, particularly in the context of food and nutritional security. Plant proteins are recognized for their importance due to health, environmental, and ethical considerations. They offer a viable alternative to animal proteins with an elevated carbon footprint and are linked with diverse health risks (Gueugneau, 2023; Singh *et al*., 2023). Notably, plant proteins can provide a complete amino acid profile and are highly digestible, making them an excellent option for meeting dietary protein needs. Similarly Soluble fibers from polysaccharides have varied functionality and are vital for human health and well-being.

Despite their potential, W/W emulsions face several limitations. Their low interfacial tension makes them susceptible to coalescence (Chen, Guo *et al*., 2021b) and sedimentation (Yang *et al*., 2020). These constraints underscore the complexity involved in stabilizing W/W emulsions and highlight the importance of understanding the underlying mechanisms to enhance their practical applications. To address these challenges, the present research aimed to develop a W/W emulsion using a combination of protein and polysaccharides and characterize the emulsions for its rheological properties, and stability.

MATERIALS AND METHODS

Materials

All the materials (Pea protein isolates (PPI) – 99.0% purity, 320 to 380 kDa., Locust Bean Gum (LBG) – 99.9% purity, 310 kDa., Guar Gum (GG) – 99.9% purity, 220 kDa., Xanthan Gum (XG) – 99.0% purity, 2000 kDa.), Fluorescein isothiocyanate (FITC) dye, Rhodamine B dye, were all analytical grade and purchased from Merck (Chennai, Tamil Nadu, India), Sigma-Aldrich Co. (Bengaluru, Karnataka, India), and deionized water was used for this research study.

Bulk solution preparation

PPI (10%), LBG (1.0%), GG (1.0%), and XG (1.0%) were prepared by adding each component (all in powder form) to deionized water at pH 6.5. The mixtures were then mixed at specific temperatures: GG and PPI at 25 °C, XG at 75 °C (Brunchi *et al*., 2016), and LBG at 85 °C (Duhan *et al*., 2021). Mixing involved using a magnetic stirrer at 600 rpm for 3-4 hours.

After mixing, the PPI solutions were centrifugated at 3350 × g (4500 rpm, Rotor - 221.55 V20, Model - Z 326 K, Hermle Co. centrifuge) at 10 °C for 20 minutes. This centrifugation step was performed to separate the sediments and extract the water-soluble protein fraction. The collected water-soluble protein fraction was then used to prepare the emulsions further. This aqueous protein fraction was further referred to as Aqueous pea protein extract.

Emulsion preparation

Ternary solutions were prepared by mixing specific amounts of bulk – Aqueous pea protein extract and LBG (PL), Aqueous pea protein extract and GG (PG), and Aqueous pea protein extract and XG (PX) solutions to obtain the required proportions with slight modifications as described (Gonzalez-Jordan *et al*., 2016). The protein and polysaccharide solutions were combined in ratios of 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, and 90:10 and using a ULTRA-TURRAX High-Speed Homogenizer, for the solutions to undergo for a homogenization process at 7000 rpm for 3 minutes (Adetunji *et al*., 2017) to create each emulsion. The formulated emulsions were transferred to screw-capped glass vials and stored in a refrigerated chamber at 4 °C.

Phase diagram

The phase diagrams (binodal) were established for biopolymer systems at pH 6.5 and 20 °C as described by (Spyropoulos *et al*., 2010). These diagrams show the regions where the biopolymer mixtures are mixed or separated into two phases (Koningsveld *et al*., 2001). Nine sets of experiments were performed for each phase diagram to obtain binodal points by analyzing the relationship between biopolymer concentrations and phase-volume ratios in binary solutions. The phase -volume ratios were plotted against weight fractions, with sufficient phase-separated systems required for a confident fit. Two binodal points were obtained from each set, providing three data points on the phase diagram – two on the binodal and one on the rectilinear diameter (which represented equal volume fractions of both phases). Each point in a diagram represented the weight/volume concentrations of the two biopolymers and the solvent (Polyakov *et al*., 1985).

Interfacial tension

The interfacial tension for the emulsions was determined using pendant drop tensiometry. Pendant drop tensiometry involves suspending a droplet of liquid from a needle and capturing its silhouette. The balance between gravitational forces and interfacial tension influences the shape of the droplet. By analyzing this shape, the interfacial tension can be determined (Berry *et al*., 2015).

Emulsion stability study

The phase separation process of the emulsion was studied by measuring the emulsions' absorbance at 247 nm using a DS-11 model Spectrophotometer from DeNovix Inc., USA. The measurements were taken at intervals of 4 h, ranging from 0 to 52 h. A 5 mL emulsion sample was taken for measurement, and the data was fitted in the exponential decay model to explain the emulsion stability behaviour.

The exponential decay model is a mathematical model that describes the process of reducing an amount by a consistent percentage over a period (Hobbie & Roth, 2006). Mathematically it is represented as exponential decay is-

$$y = A \times e^{(-kt)} \qquad \text{Eq. 1}$$

where A – Initial concentration, y – concentration after time ‘t’, k > 0 – decay constant, and t – time.

Confocal Laser Scanning Microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) measurements were done with a Zeiss LSM 710 CLSM microscope (Carl Zeiss Microscopy GmbH, Germany) utilizing an HC × PL APO 25× objective with slight modification (Michaux *et al*., 2021). A few drops of the prepared emulsion were placed on a glass slide, mounted with a cover slip, and examined. Fluorescein isothiocyanate (FITC) with emission and excitation ranges 494 nm and 521 nm, and Rhodamine B dye with emission and excitation ranges of 546 nm and 567 nm used for protein and polysaccharide phases, respectively.

Particle size determination

All emulsions were measured in triplicates after 4 hours of preparation(Igathinathane *et al*., 2008). The mean droplet diameter was reported as volume mean diameter (D_{43}).

Rheological analysis

Rheological description of the W/W emulsions was conducted using MCR 302 (Anton Paar) shear rheometer. The experiment utilized cone-and-plate geometry (CP50-2, cone diameter 50 mm, cone angle 2°, 1 mm gap) at shear rates ranging from 0.1 to 1000 s^{-1} (You *et al*., 2023).

RESULTS AND DISCUSSION

Phase diagram

A phase diagram, a binodal curve, illustrates the relationship between the components in a W/W emulsion. Fig. 1(A) to 1(C) show the binodal curves of three protein-polysaccharide combinations: PL, PG, and PX. The data point on the curve represents apparent weight fractions of protein and polysaccharide components.

Fig. 1 depicts the PL-binodal curve’s proximity to the y-axis, highlighting the PL emulsion’s superior phase stability. Emulsions with larger areas under the binodal curve had higher phase separation tendency. According to You *et al*. (2023), a distinct gap between the binodal curve and the y-axis indicated the formation of two phases. The PL emulsion’s enhanced stability over PG and PX was due to PP and LBG interactions, particularly hydrophobic bonds, which create a stable emulsion. Perrechil *et al*. (2013) explained that LBG, a polysaccharide, binds and forms a network, thereby stabilizing the emulsion and improving water retention

Table 1. Interfacial tension data of the emulsions (mN/m)

Sl. No.	Composition	PL (mN/m)	PG (mN/m)	PX (mN/m)
1.	10:90	2.52 ± 0.101	2.69 ± 0.188	2.81 ± 0.196
2.	20:80	2.39 ± 0.096	2.41 ± 0.169	2.72 ± 0.190
3.	30:70	2.13 ± 0.085	2.33 ± 0.163	2.75 ± 0.193
4.	40:60	2.06 ± 0.082	2.16 ± 0.151	2.58 ± 0.181
5.	50:50	1.97 ± 0.079	2.01 ± 0.140	2.48 ± 0.174
6.	60:40	1.78 ± 0.071	2.12 ± 0.148	2.29 ± 0.160
7.	70:30	2.55 ± 0.102	2.45 ± 0.171	2.42 ± 0.169
8.	80:20	2.86 ± 0.115	2.96 ± 0.207	2.89 ± 0.202
9.	90:10	3.24 ± 0.130	3.16 ± 0.221	3.34 ± 0.235

PL – Emulsion with phase component Pea protein and Locust bean gum; PG – Emulsion with phase component Pea protein and Guar gum; PX – Emulsion with phase component Pea protein and Xanthan gum

and strength.

The slope of Tie Line (STL) estimates in Fig. 2., representing the biphasic region's phase composition, showed that a tie line connects the phases' equilibrium compositions, with its slope indicating their compositional and volume ratio differences. The STL assesses phase proportions and emulsion stability (Spyropoulos *et al*., 2010). The line connecting rectilinear points in the PL emulsion aligns with the binodal point, signifying enhanced stability, unlike PG and PX emulsions. Other emulsion property analyses corroborate PL's superior stability.

Interfacial tension

Table 1 presents the interfacial tension depicting that PL emulsions consistently exhibit notably low interfacial tension, ranging from 1.78 ± 0.071 to 3.24 ± 0.130 mN/m. According to Wang *et al*., (2022), lower interfacial tension values indicate stronger interfacial stability, which leads to a more durable emulsion. The role of interfacial tension is vital in grasping the mechanics of phase separation, as it measures the energy needed to increase the surface area between non-mixing liquids and examine the interface's characteristics (Scholten *et al*., 2006).

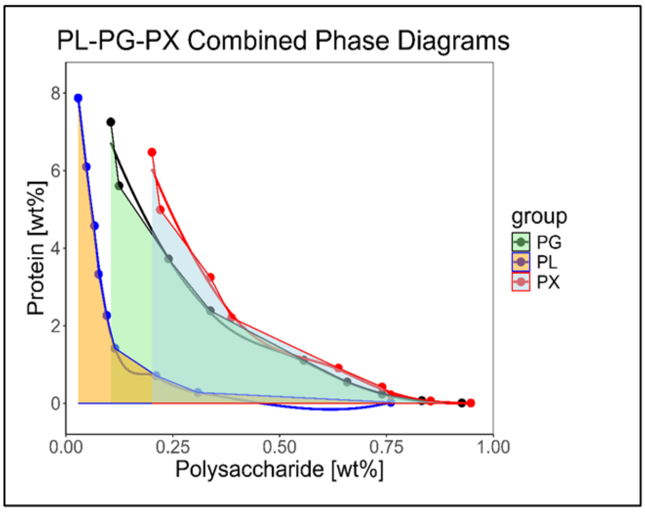


Fig. 1. *Determination of Combined Phase Diagram of PL (Emulsion with phase component Pea protein and Locust bean gum), PG (Emulsion with phase component Pea protein and Guar gum), and PX (Emulsion with phase component Pea protein and Xanthan gum)*

The interaction between proteins and LBG in PL emulsions is believed to strengthen intermolecular bonds and fully coat the interface. Introducing XG or GG into pea protein mixtures tends to dampen electrostatic forces and hydrogen bonds, creating a structure that improves water retention. When LBG levels are low, the overall negative charge decreases, leading to lower solubility and viscosity. On the other hand, higher LBG levels increase water uptake and viscosity due to more intense intermolecular hydrogen bonding, exceeding expected levels (Sánchez *et al*., 1995).

Emulsion stability study

To assess emulsion stability and phase separation time, emulsions were observed for 48 hours at 25 °C. Absorbance was measured and analyzed with the Exponential decay model, producing 'A' and 'k' values indicating initial concentration and separation rates or decay rates. From table 2, PL Emulsion showed greater stability (lower 'k') than others, indicating slower phase separation. PX Emulsions had higher initial absorbance ('A'), suggesting initial stability but faster separation due to a higher decay constant.

PX's quick phase separation, unlike PL and PG, was due to XG's net negative charge, contrasting LBG and GG's weaker charges (Chen, Beatson, *et al*., 2021) and GG is non-ionic (Grządka, 2013). This separation is caused by Segregative phase separation, where electrostatic repulsion or steric exclusion from biopolymer interactions lead to biopolymer phase separation (Jha *et al*., 2014).

Table 2. Exponential decay model parameters for each formulated emulsion

Sl. No.	Sample	A	K (s^{-1})	R^2
1	PL-1	2.583	22.320	0.89
2	PL-2	2.589	21.240	0.88
3	PL-3	2.613	20.520	0.89
4	PL-4	2.628	18.000	0.90
5	PL-5	2.659	18.360	0.92
6	PL-6	2.730	17.640	0.92
7	PL-7	2.730	19.440	0.91
8	PL-8	2.737	19.080	0.91
9	PL-9	2.776	18.720	0.94
10	PG-1	2.509	30.960	0.80
11	PG-2	2.517	25.560	0.76
12	PG-3	2.544	25.200	0.78
13	PG-4	2.541	25.560	0.76
14	PG-5	2.570	25.200	0.76
15	PG-6	2.596	21.240	0.75
16	PG-7	2.630	25.560	0.79
17	PG-8	2.627	24.480	0.80
18	PG-9	2.662	24.840	0.83
19	PX-1	2.901	23.040	0.85
20	PX-2	2.912	18.720	0.82
21	PX-3	2.936	20.160	0.89
22	PX-4	2.925	20.160	0.87
23	PX-5	2.981	18.720	0.87
24	PX-6	2.953	18.720	0.87
25	PX-7	3.047	19.080	0.89
26	PX-8	3.068	19.440	0.90
27	PX-9	3.079	17.650	0.90

PL – Emulsion with phase component Pea protein and Locust bean gum; PG – Emulsion with phase component Pea protein and Guar gum; PX – Emulsion with phase component Pea protein and Xanthan gum; A and K are model parameters – initial concentration and decay constant, R^2 –Coefficient of determination

Emulsion stability is affected by the polysaccharides used. LBG, a non-ionic galactomannan with a 1:4 galactose to mannose ratio, creates higher viscosity liquids than GG when dissolved, explaining LBG's stabilizing effect in emulsions. The pea protein and LBG blended significantly enhanced emulsion stability, especially at a 60:40 ratio. These insights are crucial for developing stable emulsions in food and other industries.

Particle size determination

From Table 3 the volume mean diameter (Dv) data of emulsions shows that PL emulsions consistently exhibited smaller particle sizes compared to PG or PX. It is well known that particle size significantly influences emulsion stability. PL-6 had the smallest particle size (0.1891 ± 0.0113 μm) attributed to its droplets' enhanced interfacial area-to-volume ratio. This ratio amplifies steric hindrance and prevents droplets from combining. Moreover, these smaller droplets' increased surface charge density promotes electrostatic repulsion, further discouraging droplet clustering. (Hossain *et al*., 2021).

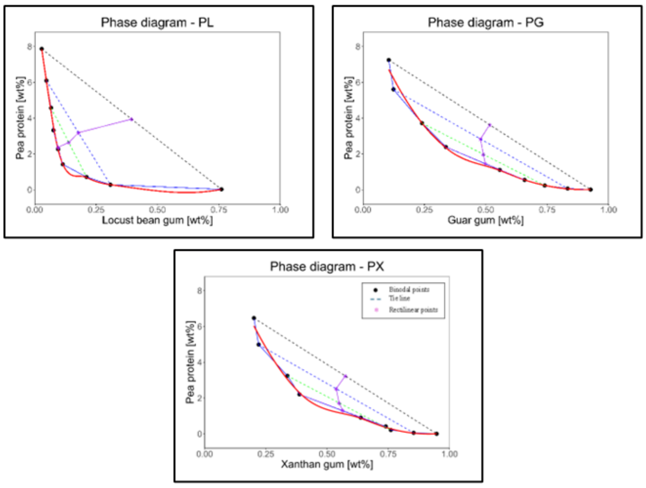


Fig. 2. *Individual Phase Diagram of PL (Emulsion with phase component Pea protein and Locust bean gum), PG (Emulsion with phase component Pea protein and Guar gum), and PX (Emulsion with phase component Pea protein and Xanthan gum)*

To summarize, PL emulsions possess more favorable particle size distributions, which suggests improved emulsion stability. These results underscore the critical influence of phase composition on particle size and emulsions' stability.

Rheological analysis

Across all three emulsions, a clear shear-thinning behaviour was observed, with PL displaying the highest viscosity, followed by PG and PX. As shear strain increased, there was a consistent decrease in both storage and loss modulus for all emulsions. A high shear modulus indicates resistance to deformation, while a high loss modulus suggests more energy dissipation during deformation. The PL combination demonstrated enhanced viscosity, storage modulus, and loss modulus, showing superior rheological properties. These findings align with similar results reported by Xiao *et al*. (2023), who studied soy protein isolate and guar gum hydrogel, further supporting the role of protein-polysaccharide interactions in enhancing emulsion stability and rheology.. Compared to PG and PX, the PL combination notably improves apparent viscosity, forming denser protein networks and coacervates. The strong interaction between PP and LBG in the PL formulation leads to better shear viscosities and microstructure, surpassing PX or PG combinations in rheological properties (Agarwal *et al*., 2023).

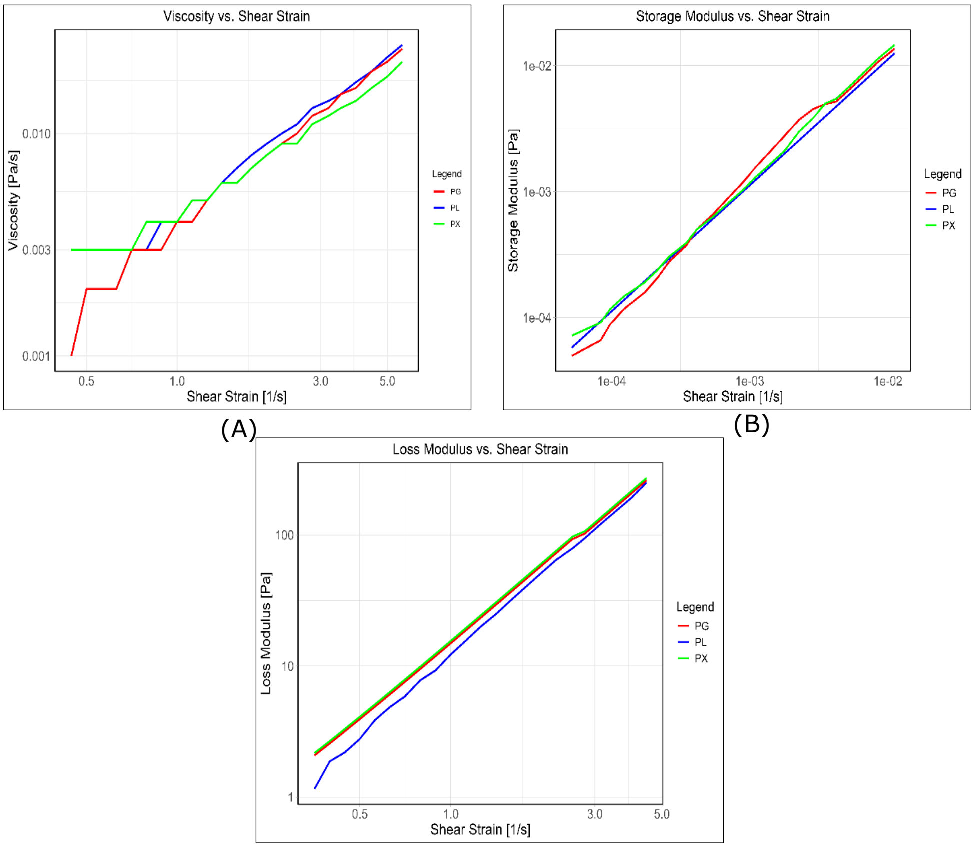
From Fig. 3 (B) and (C), it is evident that as the shear strain increased, the storage modulus decreased, reflecting reduced elastic energy conservation during deformation. The loss modulus followed a similar trend, decreasing with escalating shear strain, indicating lower energy dissipation as heat. The combination of LBG with PP shows superior storage and loss moduli enhancement, resulting in significantly improved viscoelastic properties compared to XG or GG. Further rheological studies confirm the advantageous behaviour of PP/LBG mixtures with increased storage and loss moduli. This can be attributed to the multifunctional properties of LBG, which include acting as binders, viscosity enhancers, stabilizers, and drug release modifiers (Dev Prakash & Rishi Kumar, 2023), thereby reinforcing the structural integrity and viscoelastic properties of the emulsion.

As in Fig. 3 (B) & (C), the PL emulsion exhibited lower storage and loss modulus than PG and PX, suggesting

Table 3. Particle size data (µm) of the emulsions

SL. No.	Composition	PL (µm)	PG (µm)	PX (µm)
1	10:90	0.2038 ± 0.0122	0.2975 ± 0.0178	0.3439 ± 0.0207
2	20:80	0.2052 ± 0.0123	0.2996 ± 0.0180	0.3464 ± 0.0208
3	30:70	0.2077 ± 0.0125	0.3033 ± 0.0182	0.3506 ± 0.0209
4	40:60	0.2021 ± 0.0121	0.295 ± 0.0177	0.341 ± 0.0205
5	50:50	0.1923 ± 0.0115	0.2807 ± 0.0162	0.3245 ± 0.0197
6	60:40	0.1891 ± 0.0113	0.2761 ± 0.0157	0.3191 ± 0.0191
7	70:30	0.2053 ± 0.0123	0.2997 ± 0.0180	0.3465 ± 0.0208
8	80:20	0.2111 ± 0.0127	0.3082 ± 0.0185	0.3563 ± 0.0214
9	90:10	0.2127 ± 0.0128	0.3105 ± 0.0186	0.3589 ± 0.0215

PL – Emulsion with phase component Pea protein and Locust bean gum; PG – Emulsion with phase component Pea protein and Guar gum; PX – Emulsion with phase component Pea protein and Xanthan gum



(A) (B)

(C)

Fig. 3. *Viscosity (A), Storage Modulus (B), and Loss Modulus (C) of PL-6 (Emulsion with phase component Pea protein and Locust bean gum), PG-6 (Emulsion with phase component Pea protein and Guar gum), and PX-6 (Emulsion with phase component Pea protein and Xanthan gum) as functions of shear strain*

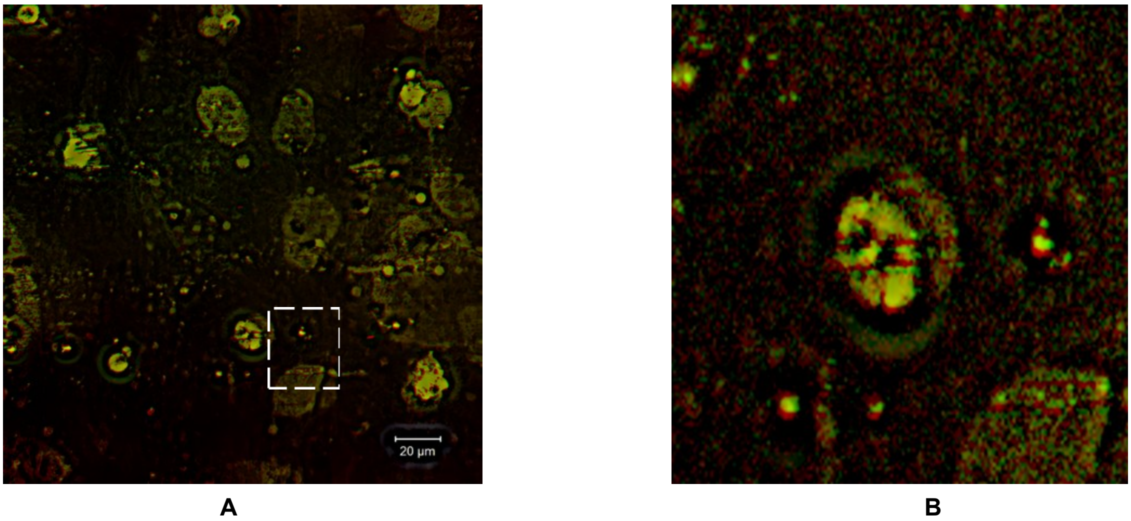


Fig. 4. *Confocal Micrographs of (A) PL-6 emulsion with 60% pea protein (6% WT %) + 40% LBG (0.16 WT %) W/W emulsion (B) Zoomed Image of PL-6 (Emulsion with phase component Pea protein and Locust bean gum) emulsion. (PP was fluorescently labelled by FTIC B (λ ≈ 546 nm) and LBG was fluorescently labelled by Rhodamine-B (λ ≈ 502 nm), respectively. Scar bar represents 20 µm.)*

less resistance to deformation and easier handling during processing. This emulsion requires less energy for deformation and may offer better stability during fluid processing, with lower chances of phase separation compared to other emulsions. These findings could have important implications for industries utilizing such emulsions.

Confocal Laser Scanning Microscopy (CLSM)

CLSM analysis of PL-6 W/W emulsions revealed tiny, spherical droplets within a continuous phase. The green FITC-labelled protein solution constituted the continuous phase, while the red Rhodamine-B-labelled LBG solution formed the dispersed phase, with yellowish interfaces indicating protein-LBG interactions. In Fig. 4(A), a protein-polysaccharide complex is highlighted and enlarged in Fig. 4(B), showing distinct green, yellow, and red areas. PL emulsions displayed a heterogeneous microstructure with varied protein-polysaccharide concentrations and an extensive protein network. The dispersed phase's varying shapes and sizes and attractive and repulsive forces between proteins contribute to the emulsion's stability, influenced by protein's higher molecular weight and lower solubility (Nunes *et al*., 2006).

Conclusion

The phase diagram analysis, interfacial tension data, and rheological behaviour collectively indicated that PL emulsions exhibited superior stability to PG and PX variants. The PL-binodal curve's proximity to the y-axis and the Slope of Tie Line (STL) estimates suggest a robust phase composition with less tendency for phase separation. The low interfacial tension in PL emulsions and the strong interactions between pea protein (PP) and locust bean gum (LBG) resulted in a stable emulsion structure. This stability was further supported by the smaller particle sizes and favourable rheological properties of PL emulsions, which demonstrate less resistance to deformation and enhanced viscoelasticity. These findings highlight the importance of component interactions and phase behaviour in developing stable emulsions, with significant implications for the food industry and other applications requiring reliable emulsion formulations. Furthermore, the use of pea protein in these emulsions supports the concept of nutritional security, offering a sustainable, high-quality protein source that aligns with global dietary needs. This study represents a significant advancement in plant-based nutrition and food technology, providing a foundation for healthier, more sustainable products, including ready-to-serve beverages and beyond.

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Conflict of interest

The authors declare that they have no conflict of interest.

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