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Special Graduation Project

Modulating the Gelling Behavior of Pea Protein (*Pisum sativum*) through Glutaminase Induced Deamidation

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Abstract

The rheological stability of pea protein products is often compromised during storage or thermal processing due to protein unfolding and aggregation. Enzymatic deamidation, which catalyzes the conversion of glutamine to glutamic acid, offers a strategy to modify the physicochemical properties of these proteins. This study assessed the effects of glutaminase PG-500 on the gelling properties of pea protein (*Pisum sativum*) by evaluating viscosity, surface hydrophobicity, particle size, zeta potential, and structural morphology. Four treatments (control, point zero two five, point ser five, point one PG-500) were tested, and data were analyzed using Origin software and a Completely Randomized Design, with significance determined via Fisher's LSD test (P < point zero five). Results showed a concentration-dependent increase in suspensions' viscosity, with point one percent PG-500 reaching point six Pa·s versus point three Pa·s for the control. The storage modulus (G') decreased, indicating a transition to a softer gel network, with a yielding point at eight percent strain before failure. Stress relaxation tests revealed faster relaxation and lower equilibrium stress in all the enzyme concentrations, suggesting a weaker gel matrix. Surface hydrophobicity and zeta potential increased, improving solubility, while particle size decreased, enhancing dispersion stability. SEM analysis revealed a less dense gel structure in treated samples. These findings demonstrate that PG-500-mediated deamidation enhances the functional properties of pea protein gels, improving their suitability for food applications while maintaining nutritional value.

Keywords: Enzymes concentrations, Gelation, Hydrophobic interactions, PG-500, SEM.

Resumen

La estabilidad reológica de los productos de proteína de guisante a menudo se ve comprometida durante el almacenamiento o el procesamiento térmico debido al desdoblamiento y agregación de las proteínas. La desamidación enzimática, que cataliza la conversión de glutamina a ácido glutámico, ofrece una estrategia para modificar las propiedades fisicoquímicas de estas proteínas. Este estudio evaluó los efectos de la glutaminasa PG-500 en las propiedades de gelificación de la proteína de guisante (*Pisum sativum*) mediante la evaluación de la viscosidad, la hidrofobicidad superficial, el tamaño de partícula, el potencial zeta y la morfología estructural. Se probaron cuatro tratamientos (control, cero punto veinticinco, cero punto cinco y cero punto uno por ciento PG-500) y los datos se analizaron utilizando el software Origin y un Diseño Completamente al Azar, con significancia determinada a través de la prueba LSD de Fisher (P < cero punto cero cinco). Los resultados mostraron un aumento dependiente de la concentración en la viscosidad de las suspensiones, alcanzando cero punto seis Pa·s con cero punto uno por ciento PG-500 frente a cero punto tres Pa·s para el control. El módulo de almacenamiento (G') disminuyó, indicando una transición a una red de gel más suave, con un punto de fluencia al 8% de deformación antes de la falla. Las pruebas de relajación del esfuerzo revelaron una relajación más rápida y un menor esfuerzo en equilibrio en todas las concentraciones de la enzima, lo que sugiere una matriz de gel más débil. La hidrofobicidad superficial y el potencial zeta aumentaron, mejorando la solubilidad, mientras que el tamaño de partícula disminuyó, lo que mejoró la estabilidad de dispersión. El análisis SEM reveló una estructura de gel menos densa en las muestras tratadas. Estos hallazgos demuestran que la desamidación mediada por PG-500 mejora las propiedades funcionales de los geles de proteína de guisante, mejorando su idoneidad para aplicaciones alimentarias mientras mantiene su valor nutricional.

Palabras clave: Concentraciones de enzimas, Gelificación, Interacciones hidrofóbicas, PG-500, SEM.

Introduction

Peas (*Pisum sativum*) are plant species belonging to the Fabaceae family, also known as legumes. These plants are annuals, growing best in cool climates, with temperatures ranging between 10 and 25 C. They are known for their spherical seeds that grow in pods and are cultivated for both human and animal consumption (T. Wallace et al., 2016; T. C. Wallace et al., 2016).

The global production of dried peas is distributed as follows: Europe produces 42.4%, America 31.9%, Asia 19.5%, Oceania two percent, and Africa four point two%, totaling 14,166,029.75 tons (FAO, 2022).

Pea protein (*Pisum Sativum*) (PP) is one of the most studied plant-based proteins due to its high nutritional value, allergen-free nature, and functional properties such as emulsification and gelation (Ishaq et al., 2022). In general, pea seeds contain 20–25% protein, 40–50% starch, and 10–20% fiber (Dahl et al., 2012). Pea protein is composed of globulins (55−65%), albumins (18−25%), prolamins, and glutelins in a lower amount (Pouzot et al., 2004).

The rheological stability of pea protein products can be challenging, especially during long-term storage or thermal processing. Heating can induce protein unfolding and aggregation, leading to changes in gel stiffness, water-holding capacity, and gel network structure (Pouzot et al., 2004). This process can also develop various molecular forces such as hydrogen bonds, ionic attractions, disulfide bonds, hydrophobic interactions, or a combination thereof (Ma & Chen, 2023; Otte et al., 1999).

Protein-based hydrogels are composed of proteins that form three-dimensional, hydrophilic polymer networks capable of absorbing large amounts of water or biological fluids. Their applications range from drug delivery systems and tissue engineering to wound healing and regenerative medicine, where their properties can be tuned for specific uses, such as enhancing cellular adhesion (Schloss et al., 2016). These hydrogels improve the textural properties of food products and can be engineered to control the release of encapsulated nutrients, thus enhancing the nutritional profile of the foods (Lima Nascimento, 2023). They exhibit significant biocompatibility, biodegradability and can respond

to various stimuli, such as deamidation making them highly versatile in multiple industries (Kalia, 2016).

Deamidation can be carried out chemically or enzymatically. Enzymatic deamidation is executed with enzymes such as peptide-glutaminase, glutaminase, and protein glutaminase (PG). Nowadays Amano 50 or 500 is derived from *Chryseobacterium proteolyticum* is the only PG known and commercially available presenting the maximum activity at a pH of five-sevenand temperatures between 50 and 60 °C (Chen et al., 2021). PG-500 deamidation is a post-translational modification resulting in the hydrolytic cleavage of the side-chain amide group of glutamine (Gln) residues in either short peptides or intact protein, but not asparagine (Asn) or free Asn/Gln. This process converts Gln into their corresponding acidic form, glutamic acid (Glu). The enzymatic mechanism involves the activation of a water molecule by the enzyme, which then attacks the carbonyl carbon of the amide group, forming a tetrahedral intermediate. The enzyme then facilitates the departure of the amine group, resulting in the formation of the carboxylate anion of the acidic amino acid. The protonation of this anion by a proton donor, such as a water molecule or an acidic residue in the enzyme's active site, yields the final deamidated product (Qu et al., 2022). Enzymatic deamidation is a critical process in protein biochemistry, as it can significantly alter the physicochemical properties, structure, and function of proteins. The introduction of a negative charge and the change in hydrogen bonding patterns due to deamidation can lead to conformational changes, affecting protein stability, folding, and interactions with other biomolecules. Additionally, deamidation can serve as a regulatory mechanism, modulating protein activity in response to various cellular signals (Reissner & Aswad, 2003).

Glutaminase-induced deamidation, facilitates structural modifications in pea protein isolates, significantly improving their textural and sensory qualities. This enzymatic process leads to the unfolding and reorganizing of protein structures, increasing the exposure of hydrophobic sites and enhancing solubility. Moreover, the process results in proteins with increased dispersibility and

suspension stability, which are essential traits for applications in liquid-based food systems (Zanardi & Alessio, 2021). Glutaminase treatment under controlled conditions such as specific enzyme concentration, temperature, and reaction time, optimizes these improvements without degrading the protein's nutritional quality, offering significant advantages for the food industry (Fang et al., 2020).

This research aimed to examine how varying concentrations of Glutaminase enzyme PG-500 via deamidation impact the gelling properties of Pea protein *(Pisum Sativum)* complementing those findings with the characterization of the physicochemical changes of those gels.

Materials and Methods

Study Location

This study was developed in the Translational Food Chemistry Laboratory at Purdue University, Nelson Hall of Food Science, 745 Agriculture Mall Drive, West Lafayette, Indiana 47907.

Materials

Pea protein isolate Green Boy (Los Angeles, California, EE. UU), Protein Glutaminase enzyme PG-500 Amano Enzyme Inc.(Naka Ku, Nagoya, Japan), sodium zero point one molar phosphate buffer solution, eight-Anilino-one-naphthalenesulfonic acid, TA Discovery HR3-rheometer, TA DIN concentric cylinder and Peltier Stainless Steel 992956 (TA Instruments, New Castle, Delaware, USA), 40.0 mm parallel plate, Peltier plate Stainless steel (TA Instruments, New Castle, Delaware, USA), Zetasizer laser Doppler Electrophoresis, Mastersizer 2000 (Malvern, Worcestershire, UK).

Suspension

Pea Protein Isolate (85%) (PPI) Green Boy (Los Angeles, California, EE. UU), was mixed with sodium zero point one molar phosphate buffer solution with pH seven in a ratio of (one:five) (w/v). The blend was homogenized (two min) stirred at room temperature (25 °C) in a hot plate stirrer (two h, 500 rpm), and submitted in the degassing process using ultrasonic equipment (20 min, 20kHz) to reduce the bubbles. The mixture with different percentages of Glutaminase Enzyme PG-500 (zero, zero point zero two five, zero point zero five, zero point one) was loaded in TA Discovery HR3-rheometer using TA DIN concentric cylinder and Peltier Stainless Steel 992956 (TA Instruments, New Castle, Delaware, USA). The established program was an oscillatory shear strain of 100/s (one h, 50 °C) to incubate PG-500.

Gels Preparation

Approximately 20 mL of the suspensions were poured into 50 mL centrifuge tubes and immediately heated at 95 °C in a water bath for 30 min, then the samples were cooled down in an iced bath for 30 min and stored at four °C overnight.

Rheological Measurements

All rheological measurements were performed using a TA Discovery HR3-rheometer (TA Instruments, New Castle, Delaware, USA).

Viscosity

The suspension was poured into the concentric cylinder. The initial temperature of 25 °C was set, increasing to 50 °C with a shear rate of 100 1/s for one hour.

Amplitude Sweep

The measure was carried out with a 40.0 mm parallel plate, Peltier plate Stainless steel (TA Instruments, New Castle, Delaware, USA). The gels were carefully placed in the base, using a temperature of 25 °C, varying from zero point one to 100% strain with a frequency of one Hz.

Oscillation Frequency

For oscillation frequency, a 40.0 mm parallel plate, Peltier plate Stainless steel **(**TA Instruments, New Castle, Delaware, USA) was also used to analyze the gels, a temperature of 25 °C was set, with an angular frequency of zero point one–15 rad/s, and a strain of zero point one percent. The results were analyzed with Equation 1, where "G'" is the storage modulus, "ω" angular frequency in a viscoelastic or viscous material, "n" is an exponent, and "K" a constant that can be experimentally determined to characterize the rheological behavior of the material under different conditions.

Equation 1

$$G' = n \log \omega + K \quad [1]$$

Stress Relaxation

The stress relaxation test was also performed at 25 °C using a 40.0 mm parallel plate, Peltier plate Stainless steel (TA Instruments, New Castle, Delaware, USA). Before recording the data, gels were soaked for 180 s, and then a strain of 2% was applied (20 min).

Creep

Creep analysis was performed with a 40 mm parallel plate. The gels were soaked for 180 s at 25 °C before stress measurement, which was executed at one Pa (15 min). The data was analyzed with Equation 2, where γ (t) is the strain and τ0 is the stress as a function of time (Dogan & Kokini, 2006).

Equation 2.

$$J(t) = \frac{(\gamma\,(t))}{\tau 0} \qquad [2]$$

Particle Size

A dilution of the initial suspension with deionized water was made to obtain a final Pea Protein (PP) concentration of five percent; the final dilution was stirred (three h, 200 rpm) and sonicated (one h, 20 kHz) and poured in the Mastersizer 2000 (Malvern, Worcestershire, UK) with a reflective index of 1.45 to measure the particle size of PP suspensions.

ζ-potential

The initial suspension was diluted with deionized water to obtain a final concentration of zero point zero five percent PP; the dilution was stirred (two h, 200 rpm) and sonicated (one h, 20kHz). The final solution was poured into the DTS1070 cell, taking care that there were no bubbles because it can have lower electrical conductivity than the surrounding liquid. This creates an electric field around the bubble, which can deflect the charged particles used to measure the ζ-potential (Clogston & Patri, 2011). The analysis was done with a Zetasizer laser Doppler Electrophoresis device (Malvern

Instrument, Worcestershire, United Kingdom). The dispersed and solution refractive index were set as one point five three and one point three, respectively, for all measurements.

Surface Hydrophobicity

Surface hydrophobicity measurements were conducted following the method outlined by (Da Chen et al., 2022). In brief, PP samples were diluted into various concentrations from zero point zer zero two five to zero point zero two percent (w/v) using a 10 mM phosphate buffer (pH seven) to obtain a total volume of 10 mL; to the final concentration, zero point zero zero one two mM eight-Anilino-one-naphthalenesulfonic acid (ANS) was added (zero point two mL total volume), stirred, and allowed to sit for 15 minutes in darkness. Subsequent fluorescence intensity measurements were taken at an emission wavelength of 480 nm and an excitation wavelength of 390 nm using a Perkin Elmer LS 55 Fluorescence Spectrometer (Beaconsfield, Buckinghamshire, United Kingdom). The relationship between fluorescence intensity and protein concentration was plotted, with the slope of this linear relationship indicating the hydrophobicity (H0) of the sample.

Scanning Electron Microscopy

Pea protein gels were fixed with zero point one molar sodium phosphate buffer (PBS) containing two point five (w/w) glutaraldehyde for 16 h. They were washed with water (three times, zero point five h) and dehydrated with series of ethanol solutions (30, 50, 70, 85, 95, and 100%) before being critical point dried. Following drying, sample blocks were cut and cracked to expose the flat surface and then sputter coated. The samples were then examined and imaged by FEI Quanta threeD FEG Dual-Beam SEM (FEI, Hillsboro, Oregon, USA) at an accelerating voltage of five kV.

Statistical Analysis

A Completely Randomized Design was followed. Four treatments were evaluated with three repetitions per treatment using Origin (OriginLab, Massachusetts, USA), through a one-way analysis

of variance (ANOVA), and mean separations with the Fisher's test to find significant differences (LSD) among samples (P < zero point zero five).

Results and Discussion

Viscosity

Viscosity is a fundamental rheological parameter that quantifies a fluid's resistance to flow, reflecting the internal friction between its molecules. It is defined as the ratio of shear stress to shear rate, offering critical insights into fluid dynamics under applied forces and its impact on protein aggregation (Macosko, 1994). Viscosity is crucial in both scientific and industrial contexts, influencing materials' processing, stability, and texture.

As illustrated in Figure 1a, all gels exhibit an initial decrease in viscosity within the first five minutes. Following this decline, gels treated with zero point zero five and zero point one percent PG-500 showed a notable recovery in viscosity, stabilizing at higher levels than those treated with zero point zero two five and no PG-500. The zero point one percent PG-500 treatment exhibited the highest viscosity, reaching zero point six Pa·s at 60 minutes, while the control showed the lowest viscosity at zero point three Pa·s, suggesting that PG-500 increases viscosity by 100%. The results established in Table 1, revealed that the viscosity in deamidated pea protein gels increases (PPG) while the PG-500 concentration increases showing statistical differences between treatments.

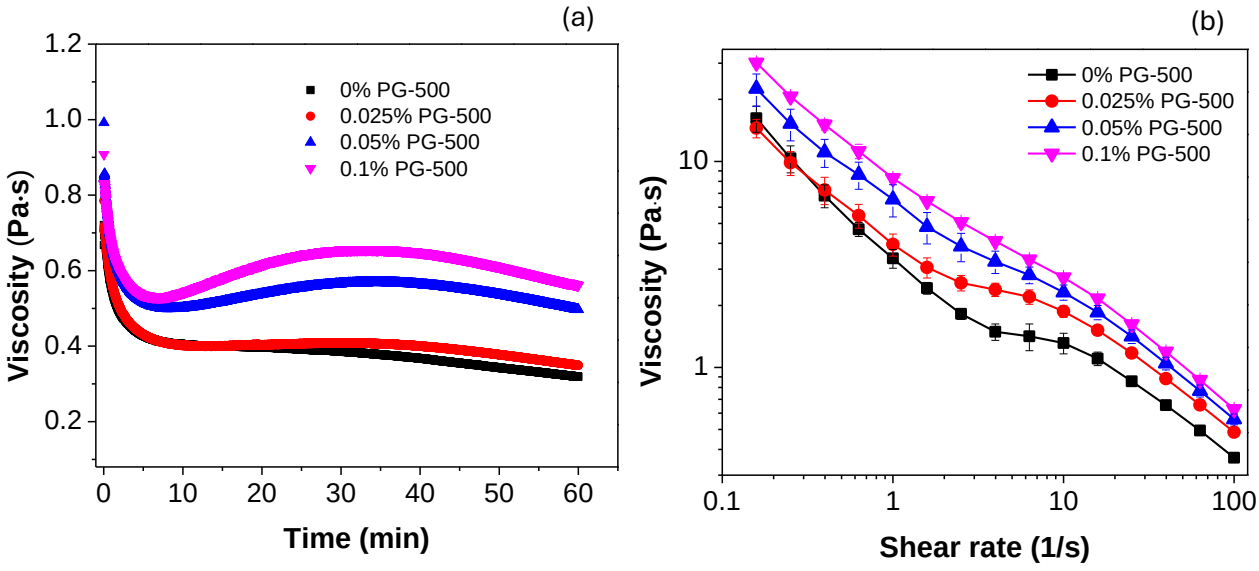
Figure 1b demonstrates a reduction in viscosity for all gels as the shear rate increases, indicating pseudoplastic or shear-thinning behavior. This behavior occurs because shear stress disrupts protein structure, causing molecules to align with the flow, thereby reducing internal resistance and decreasing viscosity ((Dharmaraj et al., 2016). Furthermore, PP viscosity increases with higher concentrations of protein glutaminase (zero point zero five, zero point one), particularly at low shear rates (three rad/s).

That can be attributed to globulins being the most abundant protein in pea protein, accounting for 55% to 65% of its composition (Lu et al., 2020). These proteins exhibit complex folding patterns that create a compact structure. Various interactions, such as hydrogen bonds and hydrophobic interactions stabilize the folding. However, this folding can be disrupted by

the removal of amide groups from glutamine residues, resulting in a gradual unfolding of the protein structure (Camilloni et al., 2016). This process allows the proteins to expand and expose more hydrophilic areas, thereby facilitating their interaction with water. As globular proteins unfold, their ability to retain water increases. This expansion of the proteins creates a more viscous environment, as water can be absorbed and retained more efficiently. This increase in viscosity is due to the formation of a three-dimensional network where water molecules become trapped between the expanded protein chains, resulting in thicker and more flow-resistant rheological behavior. Similar results were obtained in the study conducted by Wu et al. (2023) in skimmed set-type yogurt.

Figure 1

Time and shear dependent viscosity of pea protein gels containing various amounts of PG-500.



Amplitude Sweep

An amplitude sweep is a rheological test used to characterize the viscoelastic properties of materials by applying oscillatory strain or stress while measuring the material's response. The amplitude sweep aims to identify the linear viscoelastic region (LVR) (Xu et al., 2011). In the LVR, the viscoelastic parameters such as storage modulus (G') and loss modulus (G'') are independent of strain.

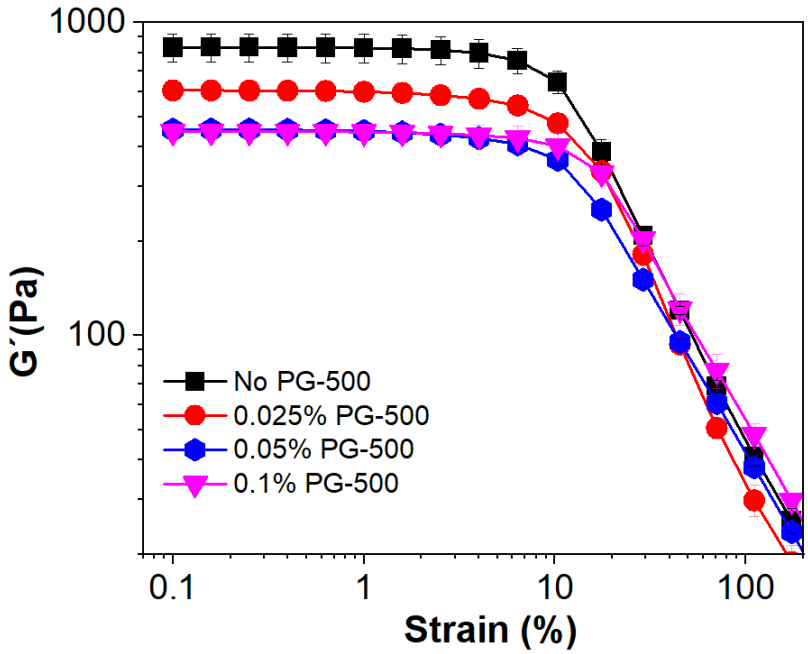
This means that measurements taken within this region reflect the material's true mechanical behavior without altering its microstructure (TA Instruments, 2004).

The maximum strain without causing permanent deformation to the PPG was 8% for all indicating the end of the LVR and the beginning of the yield point. At this point transition from a predominantly elastic to a more viscous behavior occurs highlighting a strain-induced softening behavior. This occurs since in LVR, the gels deformed elastically, with the storage moduli (G') larger than loss moduli (G"), indicating the gel-like nature of the samples. Beyond that region, G' decreased due to the breakdown of the network structure (Da Chen et al., 2021).

Moreover, the G' of the gels in the linear region decreased continuously when the concentration of PG-500 increased (Figure 2). Table 1 shows that the sample with no PG-500 has the highest G' across the entire strain range (832 Pa), indicating the highest elasticity. At zero point one and zero point zero five PG-500 treatments, G' was 448 Pa and 454 Pa respectively and did not show statistical differences between them, pointing out that G´ decreases by nearly two times at those concentrations compared with the control, suggesting the formation of softer gels network and a decrease in the elastic properties of the materials. This change in the G´ can be due to the hydrolysis of amide groups from glutamine residues in proteins, converting them to glutamic acid and releasing ammonia. This deamidation increases the net negative charge on the protein molecules, altering their conformation and intermolecular interactions. The introduction of glutamic acid residues disrupts the native conformation of proteins, causing them to unfold and expose hydrophobic regions that were previously buried, this unwinding can result in a softer, less structured gel network, reducing the G´ compactness and rigidity of the gel network (X. Li et al., 2023). Similar results were obtained for glutaminase deamidation in soybean protein isolate (Clogston & Patri, 2011; Dan Li & Xin-Huai Zhao, 2011).

Figure 2

Typical amplitude sweep, of pea protein gels containing various contents of PG-500.



Phase Angle (δ)

In rheology, the phase angle (δ) is a critical parameter used to describe the viscoelastic properties of materials. It indicates the lag between applied stress and resulting strain, representing the balance between the elastic (solid) and viscous (liquid) behavior of the material. It is defined as:

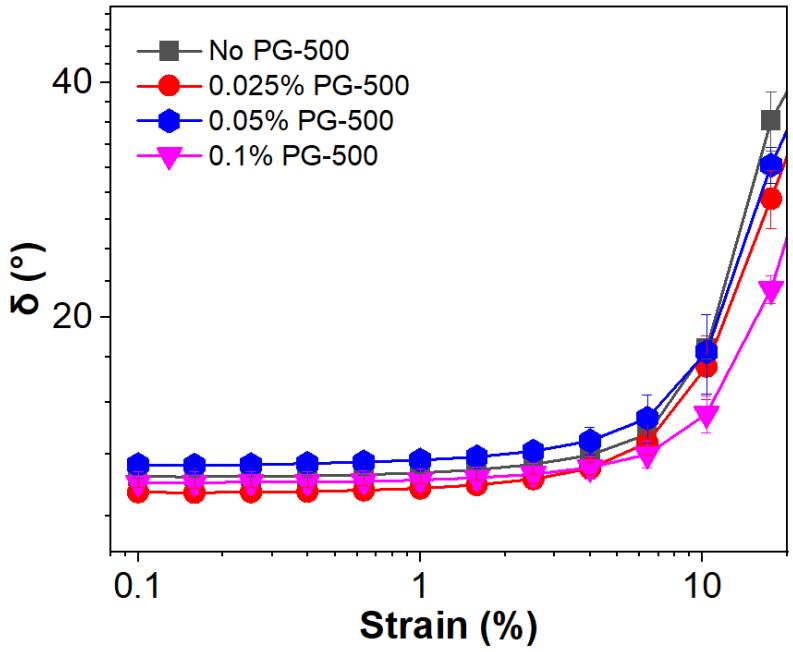
$$\delta = tan^{-1}\left(\frac{G''}{G'}\right) \quad [2]$$

A phase angle of 0° represents purely elastic behavior, where the material deforms elastically and returns to its original shape once the stress is removed. Conversely, a phase angle of 90° indicates purely viscous behavior, where the material flows and does not recover its original shape (Tabilo-Munizaga & Barbosa-Cánovas, 2005). As shown in Table 1 and Figure 3, zero point zero five percent treatment has the highest δ, indicating that PG-500 induces a more noticeable reduction in the elastic moduli of the gels. Nevertheless, all the treatments suggest a viscoelastic behavior with a tendency

toward elasticity since a low phase angle (zero- 47°) suggests solid-like behavior, characteristic of a well-structured material capable of storing elastic energy (Ramli et al., 2022). Notably, the critical strain becomes apparent from two percent, approaching a more pronounced viscoelastic response.

Figure 3

Phase angle of pea protein gels containing various contents of PG-500.



Frequency Sweep

The frequency sweep rheological test is a dynamic mechanical analysis technique used to evaluate the viscoelastic properties of materials by applying oscillatory stress or strain at varying frequencies while maintaining a constant amplitude. This method is essential for characterizing how materials respond to deformation over different timescales, which is particularly relevant for polymers, gels, and other complex fluids (TA Intrument, 2023).

Figure 4. shows that all the gels with different percentages of PG-500 increase in G´ with increasing frequency indicating that frequency and PPG elasticity are directly proportional. The control treatment (0% PG-500) showed the highest G´ increase from 437 Pa to 923 Pa in the low-frequency

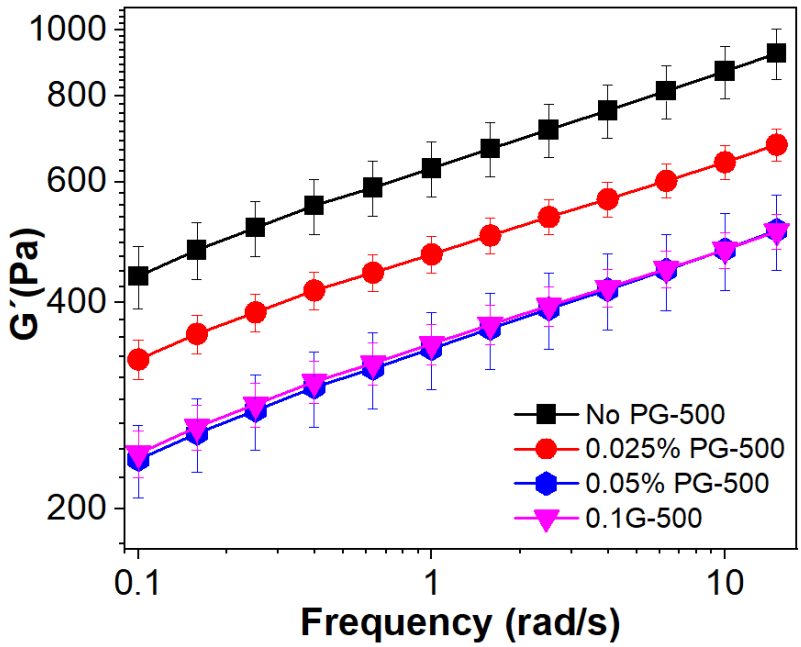
range (>10 rad/s). Table 1 suggests that the PPG treated with zero point zero five and zero point one percent PG-500 did not show statistical differences between then indicating the lowest G´, reaching 510 Pa and 508 Pa (10 rad/s) respectively. This decrease in G´ may be due to enzymatic deamidation work under physical interactions that can break down, allowing the molecules to fall off one another easily (Tang et al., 2020), (Shen et al., 2022).

To determine the kind of gel degree of frequency dependence, “n” was carried out, which is expressed by Equation 1. The “n” value calculated for each percentage of PG-500 was zero point one four which means that the concentration of PG-500 was not enough to affect the “n” value, this could be due to the magnitude of “n” in protein gels being related to the relaxation rate of the protein molecules, which is influenced by the rigidity of the polypeptide chains (Da Chen et al., 2021). Those interactions are not too affected by enzymatic deamidation.

Those gels are physical since the value of “n” is 0 for a strong chemical gel and positive (zero point one four) for a physical gel (Tang et al., 2020). Those gels are soft materials where the polymer network is formed through physical interactions rather than chemical cross-linking, allowing dynamic formation and disruption, those gels exhibit reversible solid-gel transitions and viscoelastic behavior (Tang et al., 2020), (Oscarsson & Said, 2012), (W H et al., 2001).

Figure 4

Frequency sweep of pea protein gels containing various contents of PG-500.



Creep

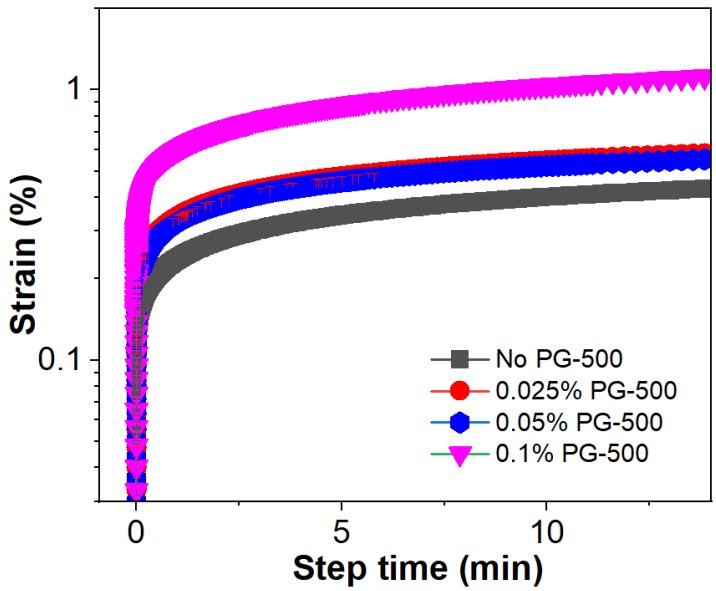
Creep rheological test is a critical method used to evaluate time-dependent deformation, it involves applying a constant load to a specimen and measuring the resulting strain over time (Dorn & Shepard, 1954). The primary objective is to understand how materials deform under sustained stress, which is crucial for predicting material behavior in real-world applications.

Figure 5 shows creep test results which can be separated into two stages, the first one is the: Primary Creep, which is characterized by a rapid rate of deformation that gradually decreases over time, the resulting strains at 0 minutes obtained in this phase were zero point one nine, zero point two six, zero point two six, zero point four nine percent for zero, zero point zero two five, zero point zero five, zero point percent PPG treated, respectively. In secondary Creep, the rate of deformation becomes relatively constant, indicating a balance between hardening and recovery processes within the material. In this stage, the compliance is calculated with Equation 2. Where zero point four four,

zero point five nine, zero point five six, and one point one three Pa^{-1} for zero, zero point zero two five, zero point zero five, zero point one percent PG-500 were the results, respectively. Table 1 indicates that the zero point one treatment showed the highest reached strain increasing the strain rate two times compared with the control, followed by zero point zero five and zero point zero two five percent concentration treatments which did not show statistical differences between them. These increased deformations and strains indicate that the presence of PG-500 leads to the formation of networks with lower density.

Figure 5

Creep curves of pea protein gels containing various contents of PG-500.



Stress Relaxation

Stress relaxation refers to the decrease in stress experienced by a material when it is held at a constant strain for a certain period. This phenomenon occurs due to the viscoelastic nature of materials, which exhibit both elastic and viscous characteristics (Whitcomb KJ, 2021).

The observed stress relaxation (Figure 6) is indicative of viscoelastic materials, which are characterized by their time-dependent strain response. Upon application of a constant strain, these materials initially resist deformation due to their elastic properties; however, over time, they undergo stress relaxation as internal friction and molecular rearrangements occur (Wittek et al., 2020). This gradual decrease in stress reflects the material's ability to dissipate energy through viscous flow while maintaining some elastic recovery.

The results reflected in Table 1 indicate that the addition of PG-500 leads to the same rate of reduction in the gel's structural integrity for zero point zero two five, zero point zero five, zero point one percent treatments, as evidenced by lower final stress values one point seven two, one point eight, and one point five three σ, respectively, in which the control, maintains the highest stress (four σ) over time. The observed relationship between PG-500 concentration and stress relaxation rates underscores the importance of network density in determining the mechanical properties of gels. With the addition of PG-500, the gel structure becomes less resilient, leading to faster relaxation times and lower equilibrium stresses. This behavior can be attributed to a decrease in entanglements and cross-links within the gel matrix as enzyme concentration rises.

Figure 6

Stress relaxation of pea protein gels containing various amounts of PG-500.

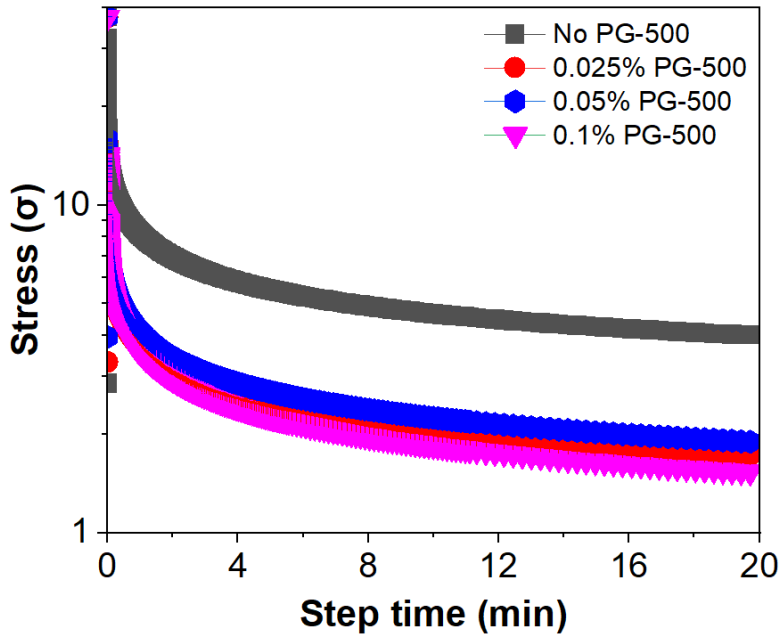


Table 1

Statistical analysis of Viscosity, Amplitude sweep, Frequency sweep, Stress relaxation, and Creep rheological tests.

Treatments	Viscosity (Pa·s)	Amplitude sweep		Frequency sweep G´(Pa)	Stress relaxation (Pa)	Creep Strain (%)
		G´(Pa)	Phase angle (°)			
Control	0.30±0.01^{D}	832.89±85.44^{A}	12.48±0.04^{B}	923.59±78.80^{A}	4.01±1.59^{A}	0.43±0.01^{C}
0.25% PG-500	0.38±0.003^{C}	605.72±35.22^{B}	11.92±0.17^{C}	679.65±37.47^{B}	1.72±0.27^{B}	0.57±0.02^{B}
0.05% PG-500	0.50±0.04^{B}	454.32±59.95^{C}	12.92±0.17^{A}	510.12±64.04^{C}	1.89±0.35^{B}	0.55±0.03^{B}
0.1% PG-500	0.61±0.03^{A}	448.22±26.47^{C}	12.26±0.38^{BC}	508.04±30.14^{C}	1.53±0.30^{B}	1.110.02^{A}
CV	0.03	0.09	0.01	0.09	0.36	0.02
Probability	<0.0001	0.0001	0.004	0.001	0.0004	<0.0001

Note. A-D:Different letters indicate the significant difference (P < 0.05) within the data in the same column; CV (%): Coefficient of variation; Pa·s: pascals per second; G´:Storage Moduluss; Pa: Pascals.

Particle Size

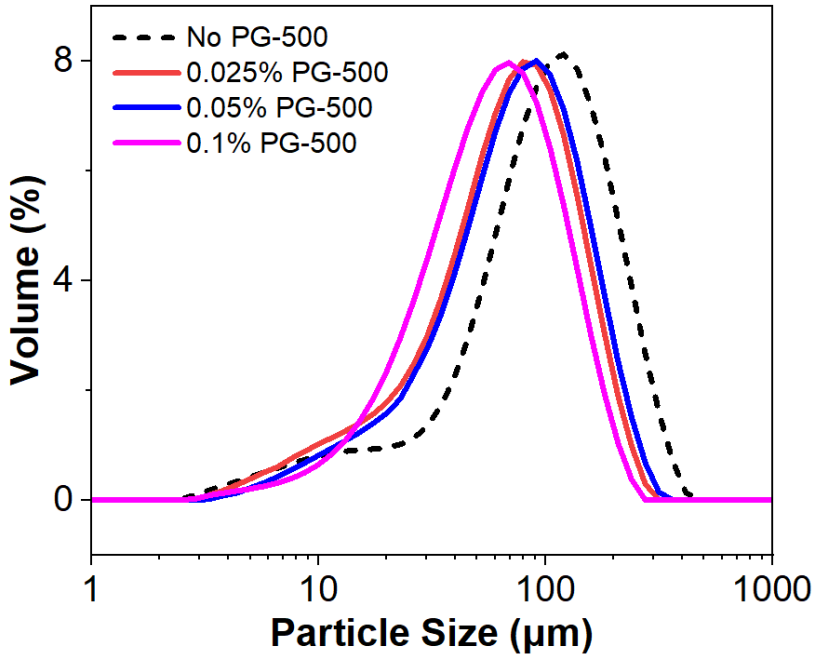
The particle size of proteins is commonly used to assess the degree of protein aggregation, which directly impacts their solubility (Y. Li et al., 2020). Smaller particles have a larger surface area-to-volume ratio, and they tend to expose more hydrophobic groups, leading to a reduction in surface hydrophobicity (Monrroy Rodriguez et al., 2021). When proteins are unfolded or denatured, this increased exposure can significantly alter surface hydrophobicity.

Figure 7 demonstrates that the addition of PG-500 reduces the particle size of pea protein (PP) suspensions, with predominant sizes of 69 µm (zero point one percent PG-500), 91 µm (zero point zero five PG-500), 79 µm (zero point zero two five PG-500), and 120 µm (control). Prior research has shown that untreated pea protein suspensions exhibit particle diameters ranging from 100 to 1000 µm, with a mean diameter of 180 ± 40 µm (Moll et al., 2021).

In PP treated with zero point one PG-500, particle sizes ranged from two point eight µm to 240 µm while the control exhibited a broader range, from two point eight µm to 479 µm This represents a reduction of up to 50% in particle size following PG-500 treatment. The decrease in particle size is attributed to the increased formation of carboxyl groups, which enhances electrostatic repulsion, weakening hydrophobic interactions and hydrogen bonding. This, in turn, leads to the dissociation of native aggregates (Chen et al., 2021). A similar reduction in particle size has been observed in other protein systems, such as skim milk and rice, upon the addition of PG-500 (Wu et al., 2023) (Hu et al., 2019).

Figure 7

Size distribution of PP suspension subjected to various degrees of deamidation.



Surface Hydrophobicity

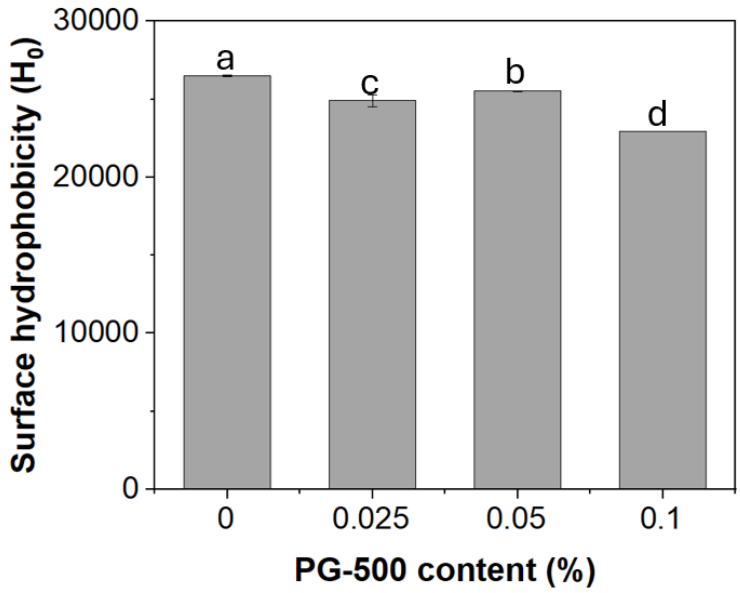
The surface hydrophobicity data, as presented in Table 2 and illustrated in Figure 8, are complemented by the findings from the Particle Size analysis (Figure 7), where surface hydrophobicity is observed to be lower at zero point zero two five percent PG-500 compared to zero point zero five. This phenomenon can be attributed to the fact that at lower concentrations (zero point zero two five percent), glutaminase is present in sufficient quantities to effectively perform deamidation without causing significant protein denaturation, thus efficiently reducing surface hydrophobicity (Kurosawa & Iwasa, 2002).

Conversely, the increase in hydrophobicity observed at zero point zero five percent PG-500 may result from non-linear interactions, where the higher enzyme concentration induces partial protein denaturation or promotes the formation of aggregates. These aggregates may expose previously hidden hydrophobic regions, leading to an increase in surface hydrophobicity.

Similarly, the subsequent decrease in hydrophobicity at zero point one percent PG-500 can be explained by the saturation of available deamidation sites. At this higher concentration, the enzyme may have a more pronounced disruptive effect on pre-existing protein aggregates, leading to the exposure of additional hydrophilic groups and the breakdown of hydrophobic structures (Wilkinson & Dalby, 2021).

Figure 8

Surface hydrophobicity of PP suspension subjected to various degrees of deamidation.



Zeta Potential

Zeta potential refers to the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle, indicating the degree of repulsion between adjacent, similarly charged particles in a dispersion (Hunter, 1988). It is a key indicator of colloidal dispersion stability. High zeta potential values (in absolute terms) imply that particles will repel each other, thus remaining in suspension and preventing aggregation.

The results of the zeta potential test, presented in Table 2 and illustrated in Figure 9, show an increase in electronegative charges as the concentration of PG-500 rises, nevertheless zero point zero two five, zero point zero five, zero point one percent PG-500 did not show significant differences between them, those findings suggest that PG-500 improves the stability of pea protein suspensions, likely due to the deamidation process, where amide groups are converted to carboxyl groups, increasing the negative charge (Hogrefe, 2010). The zeta potential for all the treatments were negative and ranged from -19 to -25 mV.

Nevertheless, a gradual reduction in the rate of increase of electronegative charges is observed. This may be due to the fact that most of the available deamidation sites are already modified at lower concentrations, and further increases in concentration do not significantly affect the surface charge (Bhattacharjee, 2016).

Figure 9

Zeta potential test of PP suspensions subjected to various degrees of deamidation.

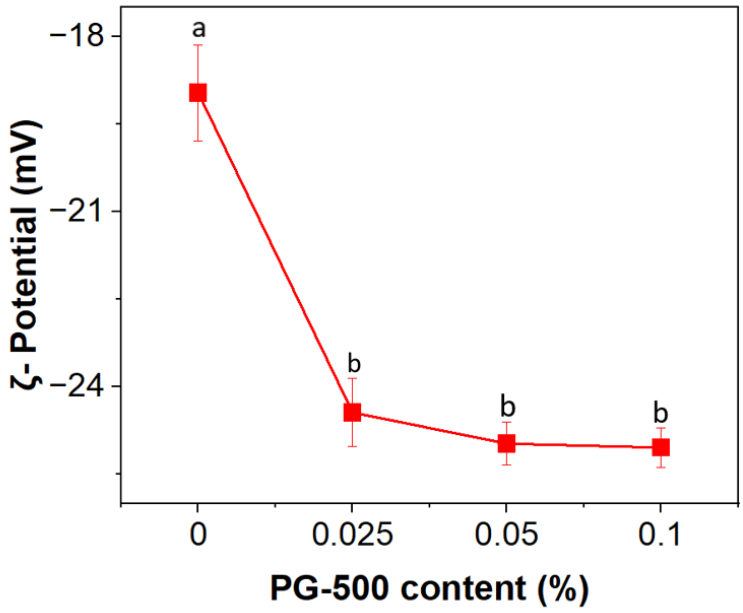


Table 2

Analysis of Surface hydrophobicity, particle size, and ζ potential in pea protein gels with different concentrations of PG-500

Treatments	Surface hydrophobicity (H_o) Media ± D.E.	Particle size (µm) Media ± D.E.	ζ potential (mV) Media ± D.E.
No PG-500	26504.50 ± 65.76^{A}	105.97 ± 2.53^{A}	-18.97 ± 0.82^{A}
0.025% PG-500	24925.00 ± 380.42^{C}	73.15 ± 1.92^{C}	-23.44 ± 0.58^{B}
0.05% PG-500	25527.00 ± 24.04^{B}	79.54 ± 2.66^{B}	-24.98 ± 0.37^{B}
0.1% PG-500	22932.50 ± 12.02^{D}	64.46 ± 1.54^{D}	-25.04 ± 0.33^{B}
CV	0.007	0.027	0.023
Probability	0.0002	<0.0001	<0.0001

Note. $^{A-D}$ Different letters indicate the significant difference ($P < 0.05$) within the data in the same column; CV (%): Coefficient of variation, H_o : Hydrophobicity index, µm: Micrometers, mV: millivolts, PG: Protein Glutaminase.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful imaging technique used to analyze the surface morphology and microstructure of materials at high magnifications. In SEM, a focused beam of electrons is scanned across the sample surface, producing secondary electrons that are collected to create detailed images. This method provides valuable insights into the topography, composition, and structural features of samples, allowing researchers to observe fine details that are not visible with conventional light microscopy (Smith & Oatley, 1955).

The scanning electron micrographs (SEM) in Figure 10 illustrate the progressive evolution of the microstructure in pea protein gels subjected to varying degrees of enzymatic deamidation. The control micrograph (Figure 10a) displays a dense, compact protein matrix characterized by a well-connected, three-dimensional network of proteins. This homogeneous structure, with minimal visible porosity, reflects strong intermolecular interactions and efficient cross-linking in the absence of glutaminase.

With the introduction of zero point zero two five percent glutaminase (Figure 10b), a slight disintegration of the protein matrix becomes evident. The micrograph reveals increased porosity and

a more open, less cohesive structure compared to the control. This suggests that enzymatic deamidation begins to cleave amide bonds, reducing network density and facilitating the formation of pores.

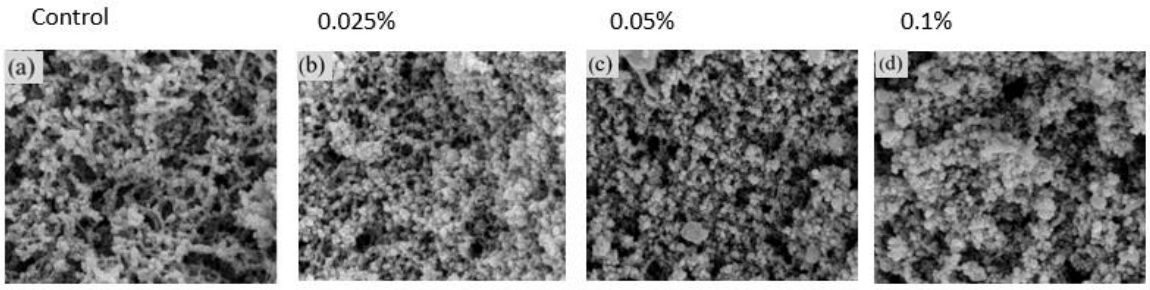
At a zero point zero five concentration (Figure 10c), the protein matrix disintegration becomes more pronounced. The micrograph shows a network with substantially higher porosity and a notably heterogeneous structure. The increased enzymatic activity leads to more extensive deamidation, resulting in a less compact, more open matrix—consistent with the progressive degradation of intermolecular bonds within the gel network.

The most extreme effects are seen with zero point one glutaminase, which reveals a highly porous and disorganized protein matrix. The structure exhibits significant disintegration, with a loosely connected, fragile network. This extensive enzymatic activity suggests near-complete deamidation of the proteins, producing an extremely open and weak three-dimensional matrix.

These observations align with previous research indicating that enzymatic deamidation enhances the solubility and porosity of legume proteins (Mookerjee & Tanaka, 2023). This study reinforces the correlation between increasing glutaminase concentrations and the disintegration of the protein matrix, demonstrating that higher enzymatic activity directly contributes to increased porosity in pea protein gels.

Figure 10

Scanning Electron microscopy of PP suspensions subjected to different degrees of deamidation.



Conclusions

The addition of PG-500 significantly increased the viscosity of pea protein suspensions by up to 100%, demonstrating a relationship between the concentration of PG-500 and the increase in viscosity.

As the concentration of PG-500 increased, a transition was observed towards a softer and less dense gel network.

The incorporation of PG-500 increased the negative charge of the protein molecules, contributing to improved solubility and a reduction in the particle size of pea protein

Recommendations

Explore a broader range of Glutaminase PG-500 concentrations beyond those tested to identify the optimal concentration that maximizes gel viscosity and stability without compromising the structural integrity of the protein network.

Conduct long-term stability assessments of the modified pea protein gels under various storage conditions (e.g., temperature, humidity) to evaluate their shelf life and functional properties over time, which is crucial for food applications.

Further research should focus on testing the modified pea protein gels in actual food formulations to assess their performance in real-world applications, such as emulsification, foaming, and textural enhancement, thereby determining their potential for commercial use in food products.

It is recommended to include additional characterization techniques, such as Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR), to gain deeper insights into the molecular changes occurring during deamidation and their effects on protein functionality.

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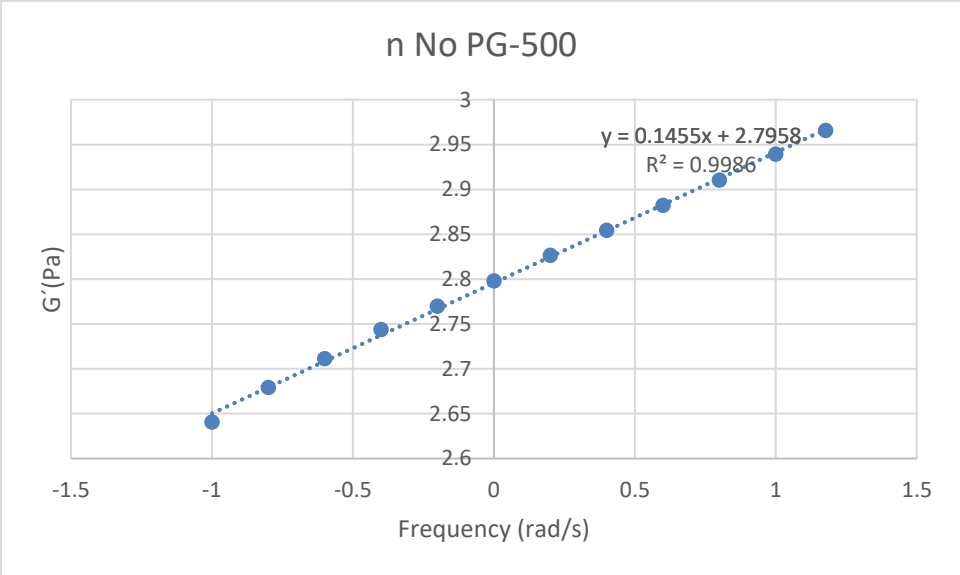
Appendices

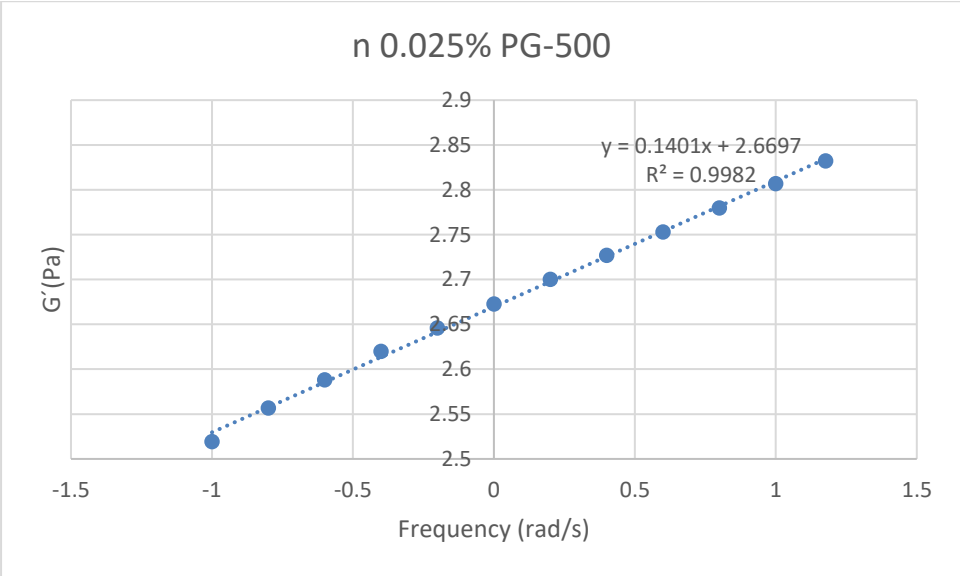
Appendix A

n value No PG-500 Frequency sweep test.



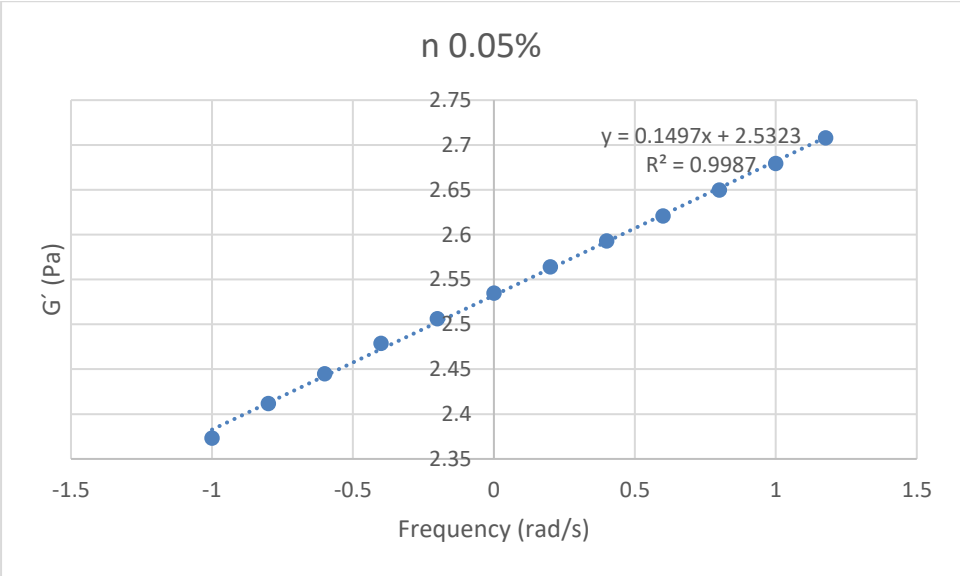
Appendix B

n value 0.025% PG-500 Frequency sweep test.



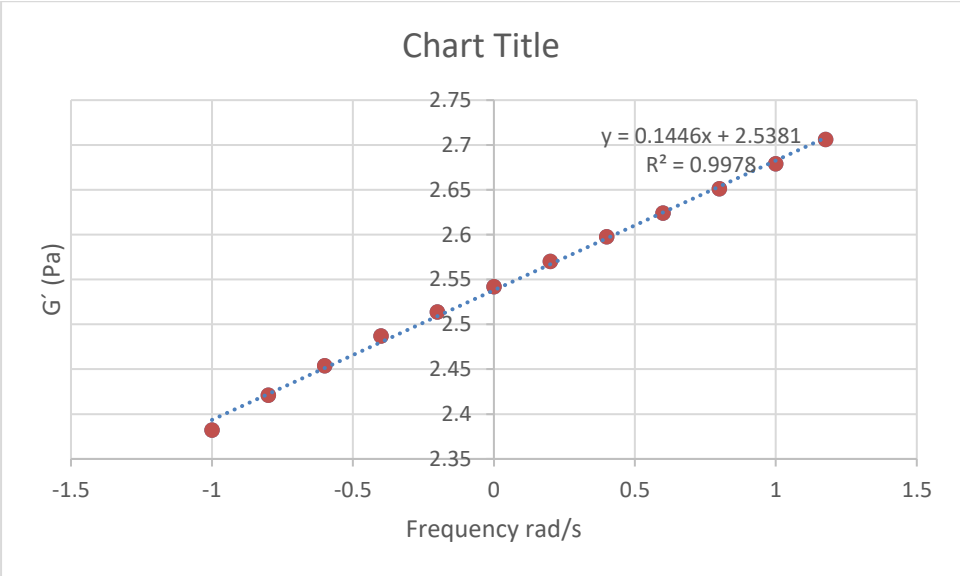
Appendix C

n value 0.05% PG-500 Frequency sweep test.



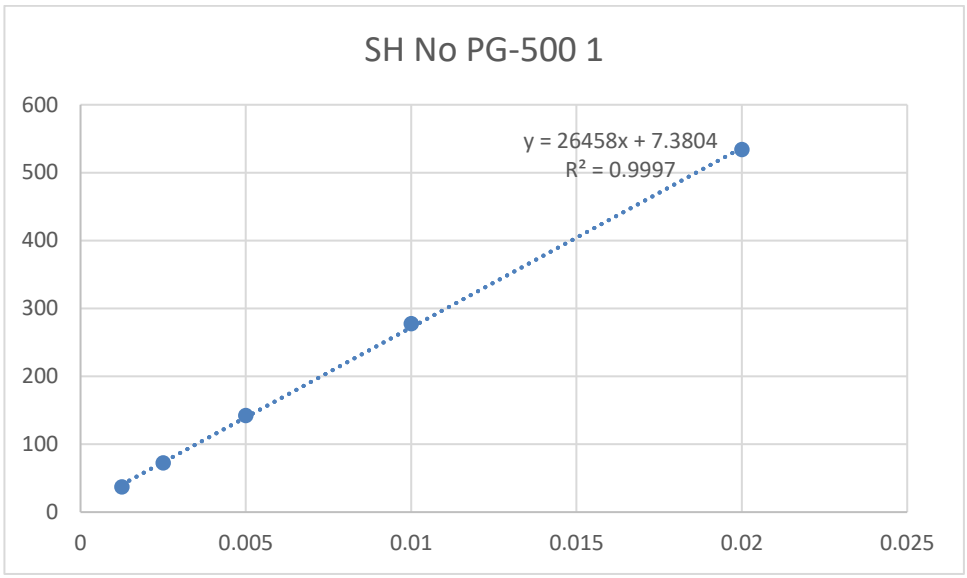
Appendix D

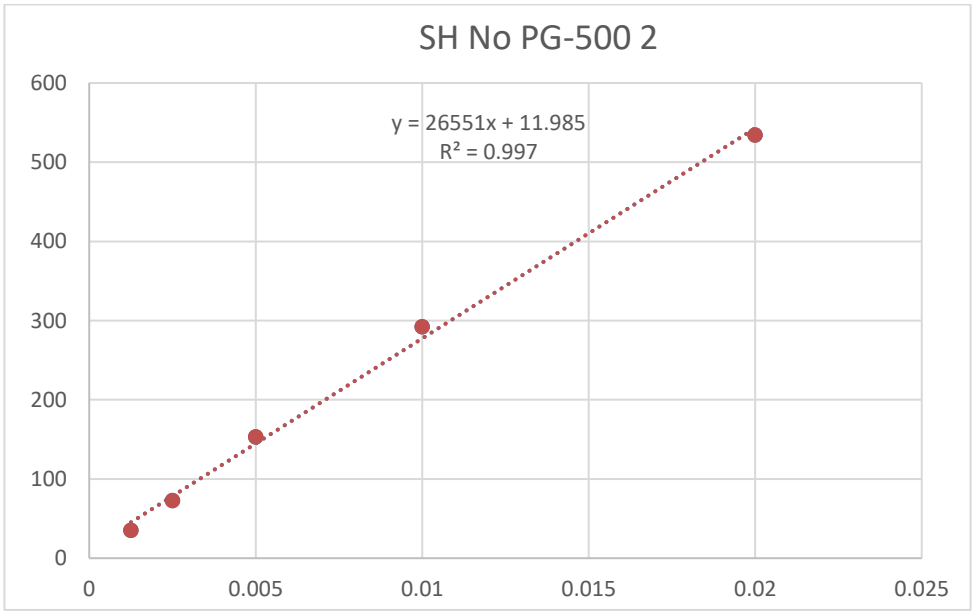
n value 0.1% PG-500 Frequency sweep test.



Appendix E

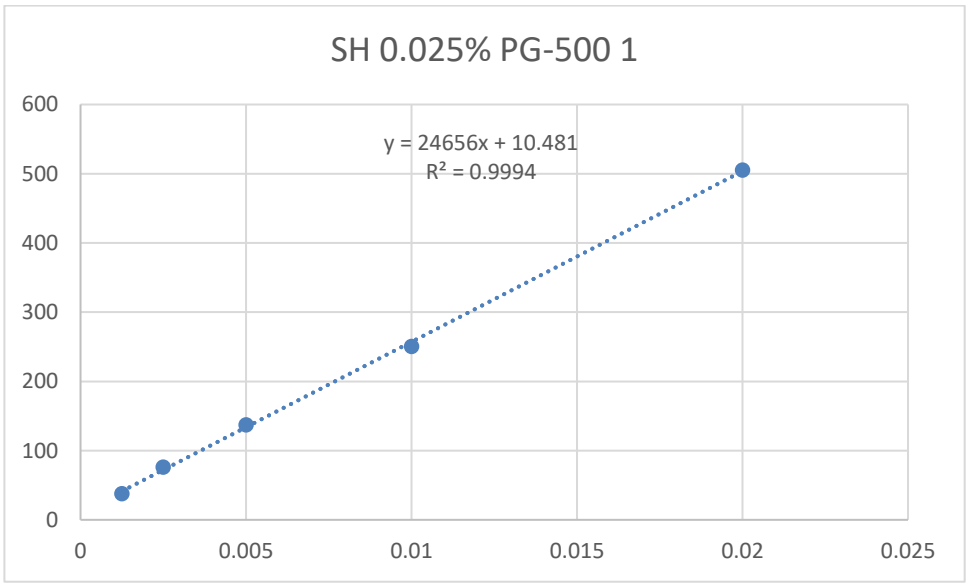
0 value No PG-500 (1,2) Surface hydrophobicity test.

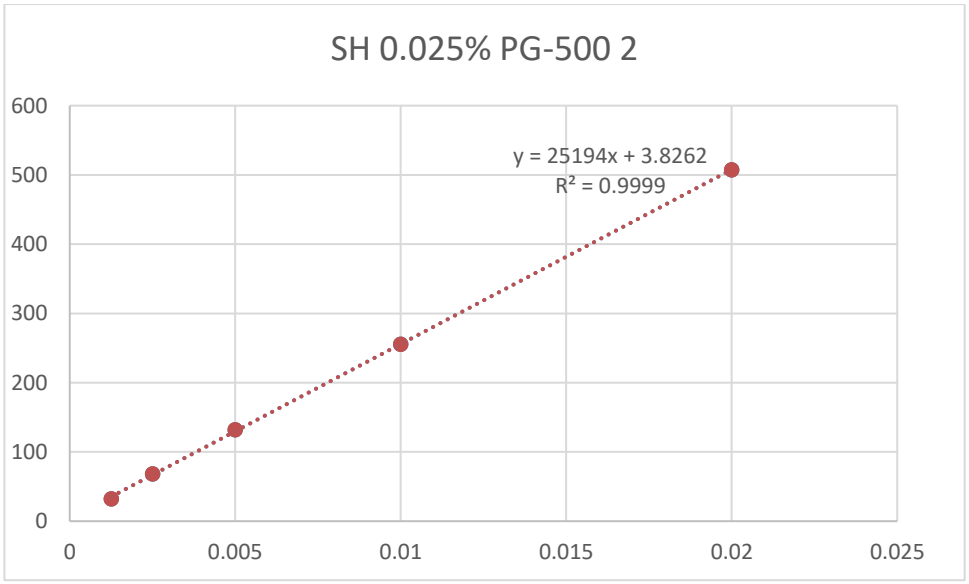




Appendix F

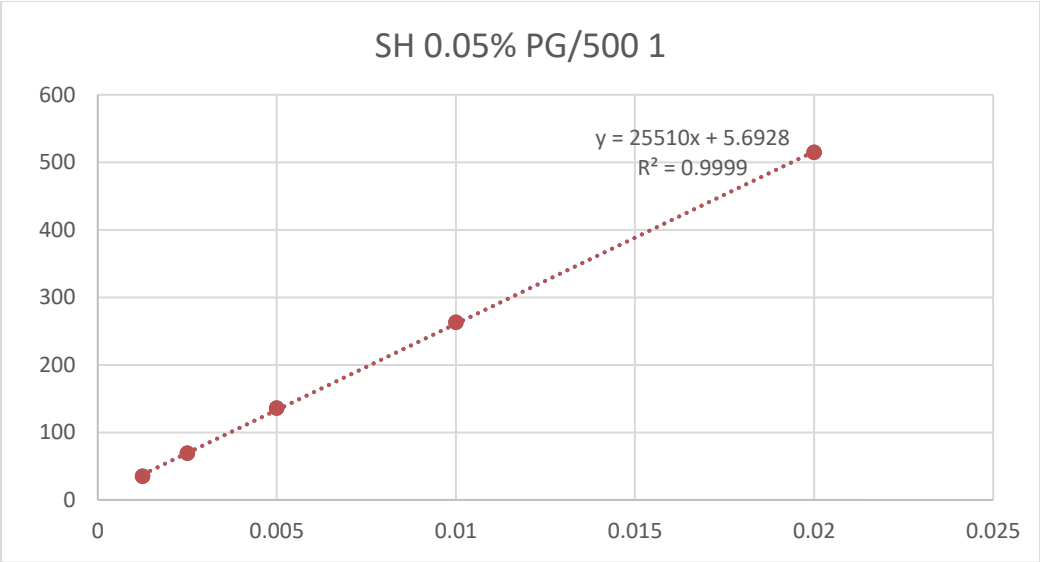
H0 value 0.025% PG-500 (1,2) Surface hydrophobicity test.

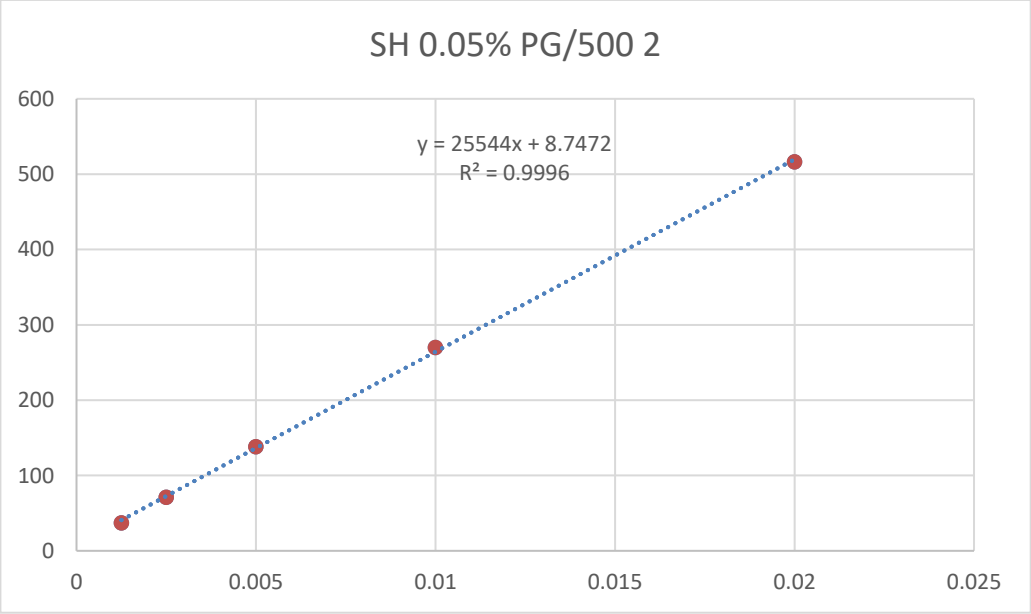




Appendix G

H0 value 0.05% PG-500 (1,2) Surface hydrophobicity test.





Appendix H

H0 value 0.1% PG-500 (1,2) Surface hydrophobicity test.