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Influence of Chitosan and Glucono-δ-Lactone on the Gel Properties, Microstructural and Textural Modification of Pea-Based Tofu-Type Product

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Abstract: This study investigated the effects of the addition of chitosan (0–1.0%) or glucono-δ-lactone (GDL) (0–60 mM) on the gel properties, microstructure, and texture of pea-based tofu-type product. Following the addition of 0.5% chitosan or 20 mM GDL, we observed a significant decrease in the hardness and cohesiveness of the tofu, resulting in a slightly discontinuous network structure with pores smaller than those in samples without chitosan or GDL. SDS-PAGE analysis revealed the induced aggregation of pea legumin (11S) and vicilin (7S) subunits (30, 34, and 50 kDa), legumin α subunit (40 kDa), and legumin β subunit (20 kDa) by chitosan or GDL. It appears that chitosan and GDL could potentially be used as food additives for the development of texture-modified pea-based tofu-type products.

Keywords: pea; chitosan; glucono-δ-lactone; gel properties; pea-based tofu

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1. Introduction

The seeds of the pea plant *Pisum sativum* L. are used in the food industry for the manufacture of food products of high nutritive value [1] and have 10–20% fiber, 40–50% starch, and 20–25% protein [2,3]. Pea plants provide a complete hypoallergenic protein, comprising two major storage fractions: the 7S (15–25%) and 11S proteins (60%). 11S (hexameric protein; 350–400 kDa) comprises six subunits (60 kDa), each of which is a combination of a basic β-chain (20 kDa) and an acidic α-chain (40 kDa). 7S is a trimeric protein (150 kDa) comprising three subunits (50 kDa) [4,5]. The isoelectric point of pea protein is pH 4.0–6.0. It is highly soluble under neutral, alkaline, and strong acid conditions. The thermal stability of 11S is higher than that of 7S, with denaturation temperatures of 92 °C and 83 °C, respectively. Pea seeds also contain phytochemicals, including glycosylated flavanols and their biosynthesis-related counterparts, which form complexes with bean proteins [6].

Peas are used to manufacture alternatives to soy-based products [7]. The preparation of soy-based tofu involves the coagulation and molding of soymilk, a colloidal solution that contains isoflavones and protein (3.6%). Roughly 80% of soymilk proteins are glycinin and β-conglycinin. Chitosan and glucono-δ-Lactone (GDL) are used as coagulants in the manufacture of soy-based tofu products [8,9]. Coagulation involves the cross-linking of soymilk proteins with coagulants, which induce the aggregation of isoflavones, glycinin, and β-conglycinin to form isoflavone–glycinin and isoflavone–β-conglycinin complexes [8].

Chitosan is obtained through the enzymatic or acidic hydrolysis of chitin, which is a positively charged polyvalent oligosaccharide composed of randomly distributed β-(1→ 4)-linked D-glucosamine and N-acetyl-D-glucosamine [10]. The interactions between soy proteins and chitosan have attracted considerable attention due to their impact on food structure. Chitosan is commonly used as a coagulant in the preparation of soy-based tofu, in which it promotes the aggregation of isoflavone–soy protein complexes [9].

Benjakul et al. [11] reported that protein–chitosan conjugates form between the reactive amino group of glucosamine in chitosan and the proteins. This suggests that the complex forms via electrostatic interaction between the carboxyl group (-COO^-) of soy proteins and the amine group (-NH_3^+) of chitosan [12]. GDL is also used as a coagulant in the preparation of soy-based tofu. GDL dissolved in soymilk is converted into gluconic acid, which lowers the pH of the soymilk to the isoelectric point, resulting in the acidic aggregation of soymilk proteins via electrostatic repulsion [8].

In the current study, the influence of chitosan and GDL was investigated on gel as well as microtextural and textural properties on pea-based tofu-type products.

2. Materials and Methods

2.1. Preparation of Pea-Based Tofu Type Product

The pea-based tofu-type product used in the current study was prepared using methods described by DePalma et al. [7] with slight modifications. Peas (200 g, *Pisum sativum* L.) were soaked in deionized water at 4 °C for 12 h. The seeds were then drained and ground with 400 mL of distilled water using a homogenizer at 32,000 rpm for 2 min. The sample was then passed through a cotton filter with a mesh opening size of 0.125 mm and the filtrate was collected. To fix the pea-based tofu-type product, various concentrations (0.1, 0.5, and 1.0%) of chitosan (molecular weight: 0.4–0.6 kDa, deacetylation > 90%; Simpson Biotech Co. Ltd., Taipei, Taiwan) or GDL (0, 20, 40, and 60 mM; Sigma Chemical Co., St. Louis, MO, USA) were added directly to the filtrate before heating the samples in a water bath at 85 °C for 1 h. The pea-based samples were then maintained at 4 °C for 12 h until the gel properties and microstructure were measured.

2.2. Assessment of Gel Properties

The texture of the pea-based tofu was measured using a TA-XT2i Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK) in accordance with the methods described by Liu et al. [13]. To measure cohesiveness and hardness, the pea-based tofu was cut into a cylindrical shape (20 mm height and 30 mm diameter), then compressed to 50% of its original height at a rate of 0.5 mm/s using a cylindrical plunger P/20 (20 mm diameter). Each sample was subjected to six measurements.

2.3. Microstructure of Pea-Based Tofu Type Product

The microstructure of the pea-based tofu was observed using SEM (TM4000plus, Hitachi, Ltd., Tokyo, Japan). Sample preparation for SEM was conducted in accordance with the methods outlined by Cao et al. [14]. The tofu was cut into cubes (10 mm × 5 mm × 5 mm) and then freeze-dried directly before being sputter-coated with gold. SEM was performed at a voltage of 15 kV with images obtained on film under 300× magnification.

2.4. Preparation of Pea Milk Samples

Pea seeds (100 g) were soaked in deionized water at 4 °C for 12 h, followed by grounding with deionized water (600 mL) before being passed through a cotton filter to collect the pea milk. The effects of adding chitosan (0, 0.1, 0.5, and 1.0%) or GDL (0, 20, 40, and 60 mM) to the pea milk (10 mL) in terms of coagulation and antioxidant activity were estimated. After incubation, the treated pea milk samples were subjected to a temperature of 85 °C for 15 min; the samples were separated into a pea milk pellet fraction (PMPF) and pea milk supernatant fraction (PMSF) via centrifugation at 5000× *g* for 20 min. The PMSF and PMPF samples were maintained at 4 °C prior to use.

2.5. SDS-PAGE and pH Analysis of PMSF

PMSF samples were analyzed in accordance with the methods outlined by Hsia et al. [8]. Briefly, the PMSF samples were analyzed using a separating gel (12.5%) and a stacking gel (5%). Each PMSF sample (0.1 mL) was mixed with sample buffer (0.2 mL, pH 6.8, 10% glycerol, 0.02% bromophenol blue, 5% β-mercaptoethanol, 2% SDS, and 70 mM Tris-HCl)

and heated to 95 °C for 5 min. A protein ladder (6 µL, 10–180 kDa) and samples (8 µL) were loaded into separate wells. After gel electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250 and scanned using an image scanner (Epson Perfection V39; Epson, Japan). The coagulation of pea proteins induced by chitosan and GDL was assessed by the magnitude of changes in the electropherogram. The pH value of the PMSF samples was measured using a pH meter (CyberScan pH500, Euctech Instruments Pte Ltd., Singapore).

2.6. Extraction and Analysis of Total Phenolic Content in PMPF Samples

The total phenolic content (TPC) of the PMPF samples with or without chitosan and GDL was measured in accordance with methods outlined by Hung et al. [15]. PMPF (0.2 g) was extracted using 80% methanol (2 mL) at 60 °C for 1 h. After centrifugation at 12,000× *g* for 15 min, the supernatant (10 µL) was mixed with 10% Folin–Ciocalteu reagent (100 µL) and incubated at 30 °C for 15 min, to which 1 M Na_2CO_3 (80 µL) was added. The absorbance at 765 nm was measured using a VersaMax™ microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). TPC was represented as gallic acid equivalents (GAE) per milliliter of PMPF extract (µg GAE/mL).

2.7. Statistical Analysis

Data analysis was performed using SAS® version 9.4 (SAS Institute, Cary, NC, USA) software, with results expressed as mean ± standard deviation. One-way analysis of variance was used to calculate the differences among treatments. All measurements were performed in triplicate, except the analysis of gel properties, which was repeated six times. Significant differences were determined at $p < 0.05$.

3. Results and Discussion

3.1. Effect of Chitosan and GDL on the Appearance and Gel Properties of Pea-Based Tofu

The gel properties of pea-based tofu with and without chitosan or GDL were investigated after storing the samples overnight at 4 °C prior to observation (arrows). Figure 1 present photos of pea-based tofu with and without 0.5% chitosan or 20 mM GDL. The appearance of samples without chitosan or GDL (control) was smoother (Figure 1A) than that of the samples with 0.5% chitosan (Figure 1B) or 20 mM GDL (Figure 1C). Lu et al. [16] reported that pea seeds contained 20–25% protein and 40–50% starch, which act as gelling biopolymers. Van de Velde et al. [17] reported that polysaccharides could alter the structure of mixed gels with a corresponding change in food texture. In our case, it was observed that the addition of chitosan and/or GDL could alter the structure of the pea-based tofu-type product.

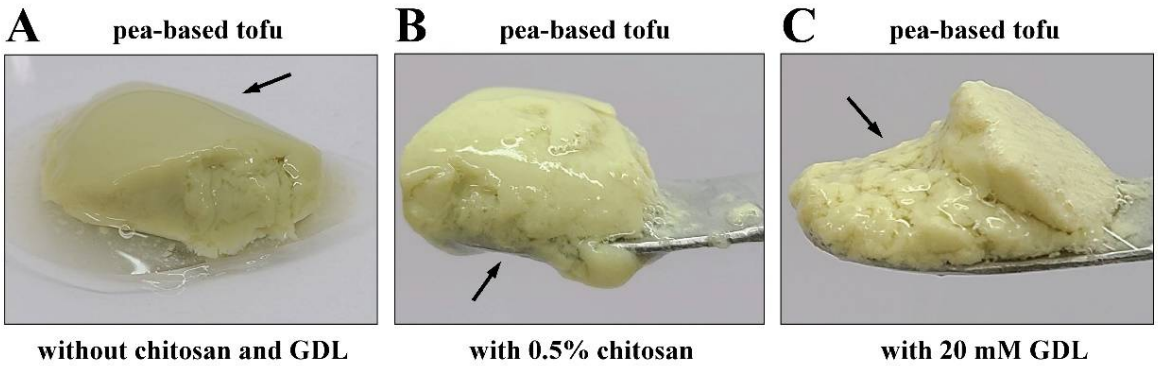

Figure 1. Appearance of pea-based tofu with and without chitosan or GDL: (**A**) without chitosan or GDL, (**B**) with 0.5% chitosan, and (**C**) with 20 mM GDL.

The influence of chitosan on the gel properties of pea-based tofu was investigated, including hardness (46.7 ± 1.2 g; Figure 2A) and cohesiveness (9.5 ± 0.2 g; Figure 2B) at 85 °C over a period of 1 h. Our hardness results were similar to those reported by

DePalma et al. [7,18]: pea-based tofu (58–98 g) and commercial soy-based tofu (51–86 g). Overall, the hardness and cohesiveness of our pea-based tofu decreased significantly with the addition of chitosan ($p < 0.05$), in the following order: 0% chitosan > 0.1% chitosan > 0.5% chitosan > 1.0% chitosan. Note that hardness and cohesiveness are the characteristics most commonly used to evaluate the quality of soy-based tofu [19]. Prabhakaran et al. [20] reported that curd formation occurred more rapidly after the addition of a coagulant, with a corresponding effect on firmness. Next, the effect of GDL on the hardness (48.5 ± 2.7 g; Figure 2C) and cohesiveness (18.3 ± 2.4 g; Figure 2D) of pea-based tofu was determined. It can be noted that these values are, respectively, 1.0 and 1.9 times higher than that of the control. We found that chitosan and GDL are used in the manufacturing of soy-based tofu products; however, neither chitosan nor GDL have been used as coagulants in the manufacturing of pea-based tofu-type products. The results showed that in the case of pea protein, chitosan and GDL perform adequately as coagulants.

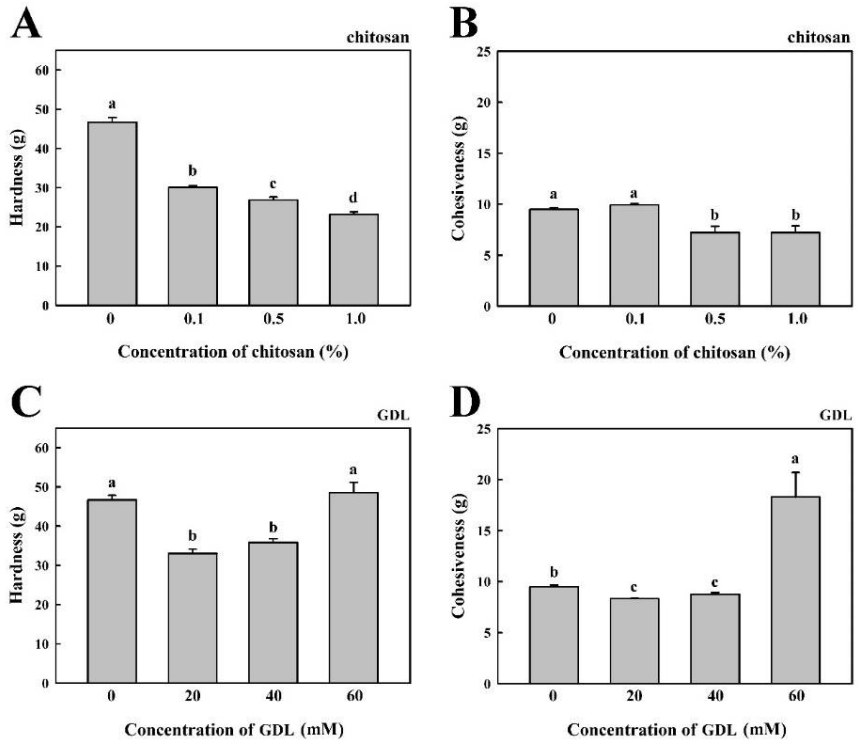

Figure 2. (**A**) Hardness and (**B**) cohesiveness of pea-based tofu as a function of chitosan content (0, 0.1, 0.5, and 1.0%); (**C**) Hardness and (**D**) cohesiveness of pea-based tofu as a function of GDL content (0, 20, 40, and 60 mM). Letters indicate significant differences at $p < 0.05$.

3.2. Effects of Chitosan and GDL on the Microstructure of Pea-Based Tofu Type Product

Figure 3 illustrate the microstructure of pea-based tofu without and with chitosan (0, 0.1, 0.5, and 1.0%). The pea-based tofu without chitosan presented a slightly discontinuous network structure with relatively large irregular pores filled with water (Figure 3A). The pea-based tofu samples with 0.1% (Figure 3B), 0.5% (Figure 3C), or 1.0% (Figure 3D) chitosan also presented a slightly discontinuous network structure with an irregular pore network (arrows in the figures), the extent of which was inversely proportional to chitosan content. Chitosan has previously been used in the preparation of soy-based tofu to modify texture [21]. Li et al. [22] reported that chitosan is tightly associated with and dispersed uniformly into a gel network.

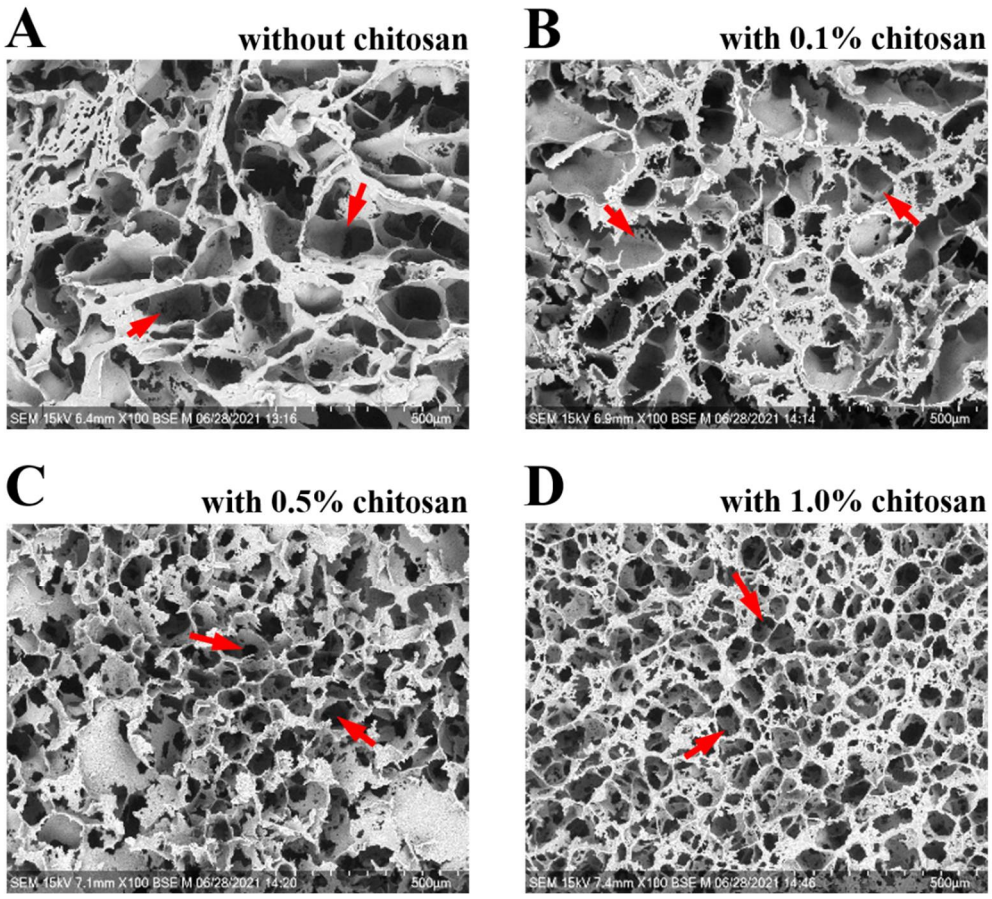

Figure 3. SEM images of pea-based tofu (**A**) without chitosan, (**B**) with 0.1% chitosan, (**C**) with 0.5% chitosan, and (**D**) with 1.0% chitosan. Red arrow: gel pore.

Figure 4 illustrate the microstructure of pea-based tofu with and without GDL (0, 20, 40, and 60 mM). The pea-based tofu without GDL presented a slightly discontinuous network structure with large pores (arrows in Figure 4A), whereas the samples with 20 mM GDL presented a similar network structure with smaller pores (Figure 4B). Yang et al. [23] reported that proteins in a GDL gel aggregate into chains, which form a compact protein network with small pores via a large number of cross-links. Hsia et al. [8] reported that in the preparation of soy-based tofu, GDL lowered the pH of the soybean proteins of the isoelectric point, resulting in the formation of a protein gel. The addition of GDL enhanced the hardness and cohesiveness of pea-based tofu in a dose-dependent manner (Figure 2C,D), and excess quantities increased porosity (Figure 4C,D). The generation of protons through the addition of GDL decreased the pH of the soymilk, which neutralized protein aggregates and led to coagulation and the subsequent formation of a three-dimensional network structure. Note that these effects can be attributed to the hydrophobic nature of the particles and the effects of van der Waals attractions [24,25]. A similar phenomenon was reported by Li et al. [26] by adding GDL to heated soymilk introduced cations, which neutralized the negative surface charge of denatured soybean proteins. They reported that the resulting aggregation of proteins into larger protein particles promoted gelation within the soy-based tofu, resulting in a loose discontinuous network structure.

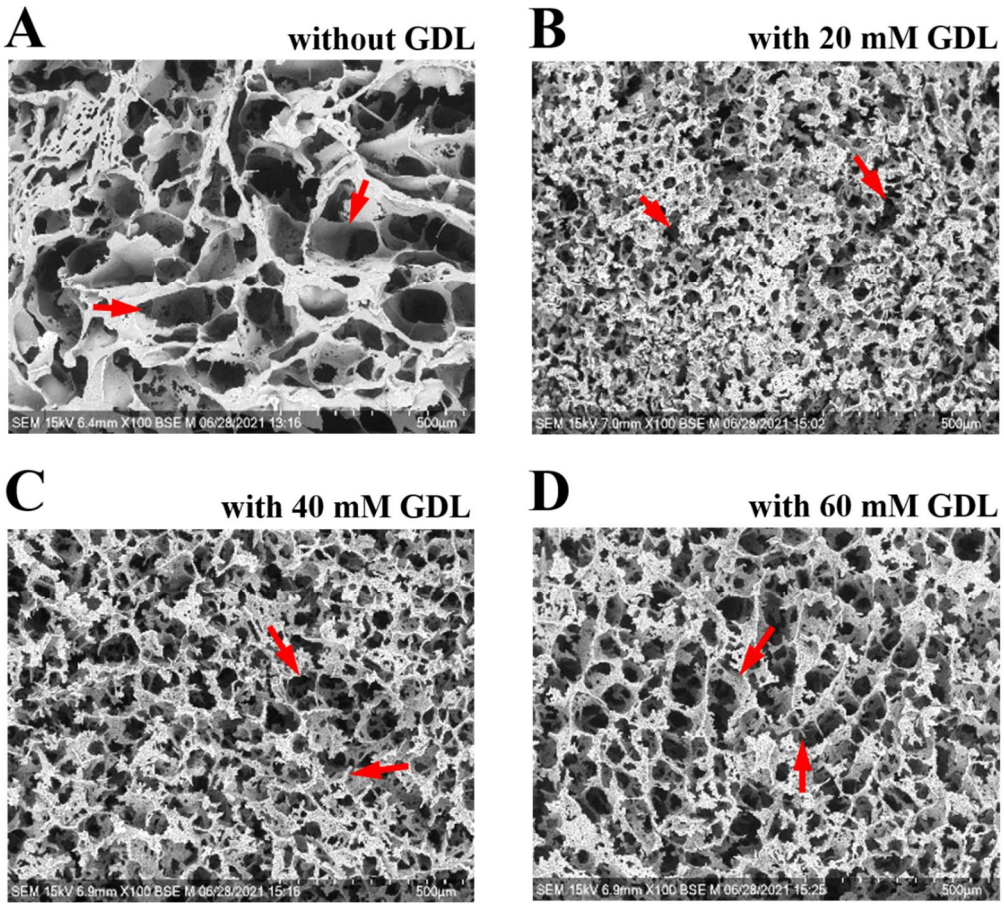

Figure 4. SEM images of pea-based tofu (**A**) without GDL, (**B**) with 20 mM GDL, (**C**) with 40 mM GDL, and (**D**) with 60 mM GDL. Red arrow: gel pore.

3.3. SDS-PAGE Analysis Showing the Effects of Chitosan and GDL on the Coagulation of Pea Proteins in PMSF Samples

Pea milk samples were incubated with various quantities of chitosan at 85 °C for 15 min, from which we extracted the supernatant. The pH of the PMSF samples decreased with an increase in chitosan content, as follows: 0 (6.6), 0.1 (6.5), 0.5 (6.1), and 1.0% (5.9). In the PMSF sample without chitosan or GDL, SDS-PAGE identified the main storage proteins as follows: vicilin (~50 kDa), legumin α subunit (~40 kDa), vicilin (~34 kDa), vicilin (~30 kDa), and legumin β subunit (~20 kDa) (see Figure 5). Okagu and Udenigwe [27] reported that the 11S proteins (350–400 kDa) comprise six acidic α-chains and six basic β-chains, while the 7S proteins (150 kDa) comprise three vicilin subunits. The concentrations of 7S and 11S proteins in the PMSF nearly disappeared in samples with 0.1%, 0.5%, or 1.0% chitosan (Figure 5A). Hsiao et al. [9] reported that the addition of 0.5% chitosan caused the aggregation of soybean proteins in soymilk. Huang et al. [12] reported that complex interactions of this nature involved hydrogen bonds between the carboxyl groups of soy proteins ($-COO^-$) and the amine groups of chitosan ($-NH_3^+$). Our results suggest that 0.5% chitosan was sufficient to cause the aggregation of 11S and 7S proteins to form pea protein–chitosan complexes, resulting in the transformation from PMSF to PMPF.

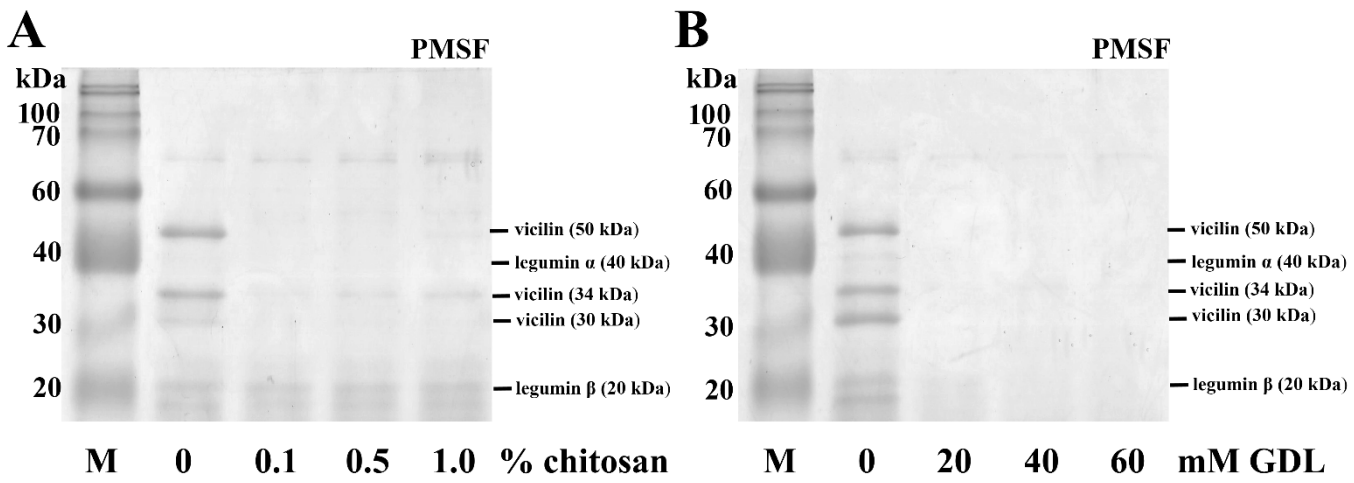

Figure 5. SDS-PAGE profiles of pea milk supernatant fraction (PMSF) following incubation at 85 °C for 15 min: (**A**) with chitosan (0, 0.1, 0.5 and 1.0%), and (**B**) with GDL (0, 20, 40, and 60 mM). M, protein markers.

Pea milk samples were incubated with various quantities of GDL (0, 20, 40, and 60 mM) at 85 °C for 15 min, from which we extracted the supernatant fraction. As shown in Figure 5B, the concentrations of 7S and 11S proteins in the PMSF nearly disappeared in samples with 20 mM, 40 mM, or 60 mM GDL. The pH of the PMSF samples decreased proportionally with GDL content, as follows: 0 (6.6), 20 (4.4), 40 (3.8), and 60 mM (3.5). Mession et al. [28] reported that the hydrolyzation of GDL led to the formation of gluconic acid, which lowered the pH and released protons, leading to a gradual decrease in electrostatic repulsion between protein aggregates. Lan et al. [29] also reported that the isoelectric point of pea protein isolate was pH 4.6. As the pH approached the isoelectric point, the solubility of the pea proteins decreased, leading to the precipitation of protein agglomerates [30]. Thus, our results suggest that the GDL aggregated the 11S and 7S proteins from PMSF to PMPF, as indicated in the pellet fraction.

3.4. Effects of Chitosan and GDL Addition on Total Phenolic Content in Pea-Milk Pellet Fraction

As shown in Figure 6A, the total phenolic content (TPC) of PMPF samples increased proportionally with the quantity of added chitosan ($p < 0.05$), as follows: 0% (43.0 ± 0.6 μg/mL), 0.1% (51.1 ± 2.0 μg/mL), 0.5% (52.3 ± 2.0 μg/mL), and 1.0% (54.4 ± 0.6 μg/mL). As shown in Figure 6B, the TPC of PMPF samples increased proportionally with the quantity of added GDL, as follows: 0 mM (43.0 ± 0.6 μg/mL), 20 mM (44.0 ± 0.8 μg/mL), 40 mM (47.6 ± 0.6 μg/mL), and 60 mM GDL (47.3 ± 0.2 μg/mL) (Figure 6B). As mentioned above, the phenolic compounds contained in pea seeds possess redox properties and are responsible for antioxidant activity [6]. Note that these phytochemicals can form complexes with bean proteins. These results suggest that the phenolic compounds that bonded to pea proteins formed complexes, which underwent coagulation when 0.5% chitosan or 20 mM GDL was added to the PMPF.

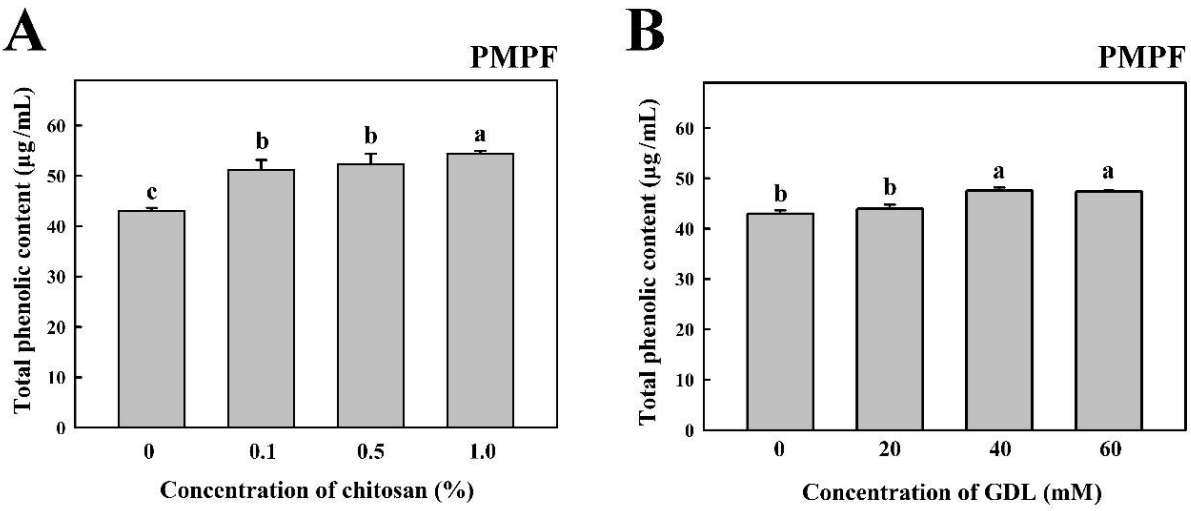

Figure 6. Effects of chitosan (0, 0.1, 0.5, and 1.0%) or GDL (0, 20, 40, and 60 mM) on the TPC in pea milk pellet fraction (PMPF). (**A**): chitosan. (**B**): GDL. Different letters indicate significant differences ($p < 0.05$).

4. Conclusions

In this study, the effects of chitosan and GDL on the gel properties, microstructural, and textural modification of pea-based tofu-type product was investigated. Our results indicated that pea tofu was formed by heating pea milk without chitosan or GDL to 85 °C for 1 h and that the addition of chitosan or GDL reduced the hardness and cohesiveness of the tofu. The resulting pea-based tofu presented a slightly discontinuous network structure with pores smaller than that of tofu without chitosan or GDL. In conclusion, chitosan and GDL could be practical food additives for developing texture-modified pea-based tofu-type products.

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