

## Protein-Phenolic Interactions in Food

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### Abstract

Interest in protein-phenol interactions in biological systems has increased substantially during the past two decades. More recently, there has been particular interest in protein-phenol interactions in food systems, as a result of widespread reports on the roles of phenolic compounds in nutrition and health. Many phenolic compounds are now recognized for their nutraceutical properties. The biochemical, nutritional and immunological properties of these phenolic compounds, can be associated with their relationship with certain proteins. A good example is the recent recognition of soybean proteins containing isoflavones, for their health benefits and for prevention of certain diseases. In order to understand protein-phenol relationships, it is essential to determine the nature of the chemical/physicochemical interactions between the proteins and the biologically active phenols. The overall objective of our study was to use a model system to investigate the mode of interaction between selected food proteins and phenolic compounds. Bovine serum albumin (BSA) and soybean glycinin were used with two phenolic compounds: Gallic acid (3, 4, 5-trihydroxybenzoic acid) and biochanin-A (5, 7-dihydroxy 4-methoxy isoflavone). The interactions were investigated at incubation temperatures of 35°C, 45°C and 55°C at pH 5, 7 and 9. SDS and Native polyacrylamide gel electrophoresis (PAGE), differential scanning calorimetry (DSC), and Fourier transform infrared (FTIR) spectroscopy were used to identify protein-phenol interactions. Certain phenolic compounds combined with BSA resulting in higher migration bands and prevention of protein aggregation. In general, the thermal stability of the proteins increased as a result of interaction with the phenolic compounds. The interaction of the phenols with the proteins resulted in changes in protein secondary structure. The most pronounced effects were observed with gallic acid, while the least effects were observed with the isoflavone biochanin-A.

### Keywords:

Isoflavones, BSA, SDS-PAGE, FTIR, DSC, phenolic compounds

### 1. Introduction

The role of food proteins in nutrition and health has long been well established. More recently, the effect of phenolic compounds present in foods is being widely studied. Consequently investigations on protein-phenolic interactions are the study of interest in many areas of food research. Phenolic compounds are constituents of plants, including many that are consumed widely by humans a good, example is dietary Tannins. Data from humans, and animal studies suggest that dietary phenolic compounds play important roles in prevention

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against a wide range of diseases including cardiovascular diseases, certain types of cancers, and osteoporosis, however they can also be harmful (Haslam, 1998).

Soybean besides being excellent source of protein (35-40%) and fat/oil (15-20%) also contains several isoflavones as a group of phenolic compounds (Messina, 1999). Soybean isoflavones have been reported to confer significant health benefits. The interest in soybean isoflavones has become intense to an extent that recently two international conferences have been convened to report the state of knowledge in the field. In October 1999 Food and Drug Administration (FDA) of United States responded to the petition by Protein Technologies International, by authorizing the claims that soybean is good for heart function. According to FDA, intake of 25g soybean protein a day can reduce the risk of heart diseases when used with low fat diet, the 25g soybean protein recommended by FDA contains 25mg of isoflavones. The isoflavones in soybean can be strongly attached with proteins in the seed (Palevitz, 2000).

Protein-phenolic interactions have been categorized as either reversible or irreversible. Reversible protein-phenolic interactions lead to the formation of insoluble complexes in solution; however, entirely new product can result from irreversible reaction (Haslam et al, 1999). Tightly coiled, globular proteins show less affinity for phenolic compounds as compared to proteins that have random coil or loose conformation (Hagerman, 1989) and in particular those which are rich in amino acid proline (proline rich proteins- PRP). Both the aromatic nuclei and the hydroxyl groups of the aromatic ring of phenolic compounds provide the principal binding sites for the protein-phenolic complexation (Murray et al, 1994). Hydrogen bonding and hydrophobic interaction have been found the primary attractive forces between protein molecule and phenolic group (Bartolome et al, 2000).

Some of the methods used to investigate interactions, for a range of proteins and phenolic compounds are:  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy, microcalorimetry, equilibrium dialysis, and enzymes kinetics and inhibition. However, examining these effects we have selected three different analytical techniques: polyacrylamide gel electrophoresis (PAGE), differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). These techniques have been found useful for studying various parameters on proteins in aqueous solutions. The objective of this investigation was to evaluate the potential of the above analytical techniques in studying protein-phenolic interactions. The findings can further reveal the availability of isoflavones in biological system compared to simple phenols.

For the purpose of our study we selected Bovine Serum Albumin (BSA) which is recognized as the industry standard for protein quantitation and soybean glycinin as investigational protein known to carry isoflavones. These 2 proteins will be treated with Gallic Acid, a simple phenol, and Biochanin-A an O-methylated isoflavone, as a complex phenol

## **2. Material and Methods**

### **2.1. Materials**

Bovine serum albumin (BSA) (product A-2153) was obtained from Sigma Chemical Co (St. Louis, MO, USA) and used as received. Cryoprecipitated soybean glycinin previously prepared in our laboratory (Ramadan, 2000) and stored at  $-20^\circ\text{C}$ , was used. Gallic acid (3, 4, 5 trihydroxybenzoic acid, product G-7384) and biochanin-A (5, 7 dihydroxy 4' methoxy isoflavone, product D-2016) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The phenolic standards were stored at room temperature.

## 2.2. Preparation of samples for protein-phenolic interactions

The solution of proteins (BSA and soybean glycinin) and phenolic standards (gallic acid and biochanin-A) were prepared in phosphate buffer at pH 5, 7, and 9 by dissolving 6 mg protein in 6 mL of each buffer in 15 mL centrifuge tube. The concentration was increased eight-fold (8X) times for soybean glycinin, compared to the concentration of BSA. The phenolic standards were prepared by dissolving 3 mg in 3 mL of respective buffers. The phenolic buffer solutions were heated to 100°C in a water bath for 5 min to facilitate solubility and then cooled to room temperature before incubating with proteins. Proteins and phenolic compounds were mixed at 1:1 ratio in a 15 mL centrifuge tube. The final concentration of each reactant in the solution was (0.05% W/V). For the control, each centrifuge tube contained 1 mL protein and 1 mL of the corresponding buffer. The samples were incubated in a water bath at desired temperature of 35°C, 45°C and 55°C for 2 hr, and then cooled to room temperature.

Due to the high concentration of soybean glycinin, it was not possible to completely solubilise this protein in the buffer solutions. Results from incubation temperature of 55°C for electrophoresis are reported. Further, heat treatments were not required for DSC and FTIR experiments.

### 2.2.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini-protein II electrophoresis cell unit. All chemicals were purchased from Bio-Rad (Mississauga, Ontario, Canada, electrophoresis grade).

### 2.2.2. Sample preparation for SDS-PAGE

From the incubated solutions 262  $\mu\text{L}$  (0.131 mg BSA) was diluted with 300  $\mu\text{L}$  of sample buffer (3% SDS, 0.7 M 2-mercaptoethanol, 25 mM Tris-HCl pH 6.8, 1% glycerol and 0.05% bromophenol blue) in a microcentrifuge tube. Samples were heated at 95°C for 5 min in a water bath. The final concentration of the protein reached 0.23 mg mL<sup>-1</sup> (0.23%).

## 2.3. Concentration and preparation of gels

Separating gels of 10% concentration and 6.5% concentration stacking gels were used. Separating gels containing 0.1% (W/V) SDS, 16% (V/V) glycerol and 0.375 mol L<sup>-1</sup> Tris HCl (pH 8.8); polymerization were initiated by the addition of 0.25% (W/V) ammonium persulphate (APS) and 0.05% N, N, N', N' tetamethylethylene diamine (TEMED). The 6.5% stacking gel included 0.1% SDS and 0.25 mol L<sup>-1</sup> Tris-HCl (pH 6.8). Polymerization was catalyzed by the addition of 0.1% APS and 0.1% (V/V) TEMED. After mixing the appropriate amounts of the reagents, the gel solutions were poured between 7 × 8 mm glass sandwich plates for polymerization. Before pouring the stacking gel, combs were mounted on the top of the gels to form 10 sample wells. The holding capacities of individual gels were approximately 25  $\mu\text{L}$ . After polymerization the combs were removed and the sample (15  $\mu\text{L}$ ) was applied to the gels. The upper buffer chamber was then immediately filled with approximately 115 mL of running buffer (50 mmol L<sup>-1</sup> Tris-HCl, pH 8.3, SDS 0.1% and 1.384 M glycine), until the buffer reached a level halfway between the short and long plates. The remainder of the buffer was poured into the lower buffer chamber so that at least the bottom 1 cm of the gels was covered.

## 2.4. Sample loading

The solution of proteins (BSA and soybean glycinin) and phenolic standards (gallic acid and biochanin-A) were loaded into the sample wells using a Hamilton syringe. Broad range molecular weight protein standards were also loaded on the gels.

## 2.5. Running conditions

The electrophoretic run was performed at a constant current of 15 milliamps (mA) per gel. The voltage and power limits were 350 V and 15 W respectively. To maintain the temperature at 20°C the bottom part of the electrophoresis unit was immersed in a water tub. The power supply was terminated when the tracking dye front (bromophenol blue) reached the end of the gel; this took 1 hr and 17 min.

## 2.6. Protein Fixing, Staining and destaining solutions

At the completion of the run, the gels were removed from the glass plates and immersed in a fixing solution (20% V/V methanol, 10% V/V acetic acid, and 70% distilled water) for 2 hr. The fixing solution was discarded and the protein bands were stained with 10% (V/V) acetic acid, 20% methanol (V/V) and 0.1% (W/V) Coomassie Brilliant Blue R250 for 10 hr. Destaining was accomplished by placing the gels in the fixing solution repeatedly until the back ground color was completely removed. The gels were stored in 7% acetic acid in a refrigerator till they were photographed.

## 2.7. Native Electrophoresis

### 2.7.1. Sample preparation for Native-PAGE

A total of 612  $\mu\text{L}$  from the incubated solution, containing (0.306 mg of BSA) was mixed with 300  $\mu\text{L}$  of sample buffer (0.3 mol  $\text{L}^{-1}$  Tris-HCl, pH 8.8, 10% glycerol and 25 mg  $\text{mL}^{-1}$  bromophenol blue) to give a final protein concentration of 0.33 mg  $\text{mL}^{-1}$  approximately 0.033 %.

### 2.7.2. Concentration and preparation of gels

A 10% separating and 6.5% stacking gel were used. Polymerization was initiated by the addition of 0.25% (W/V) APS and 0.05% TEMED for separating gel and 0.1% (W/V) APS and 0.1% TEMED for stacking, respectively. Gel preparation and sample loading were similar as described for SDS-PAGE.

### 2.7.3. Electrophoresis Condition

The running conditions, power limits and temperature were similar to those described for SDS-PAGE, except that SDS was not the part of running buffer. A constant current of 7.5 mA per gel was applied. The time of the electrophoretic run was 2 hr and 45 min for BSA and 3 hr and 30 min for soybean glycinin.

### 2.7.4. Protein fixing, staining and destaining solutions

Protein fixing, staining and destaining was performed as described for SDS-PAGE.

## 2.8. Differential Scanning Calorimetry (DSC)

A DSC equipped with TC 11 processor (Mettler TA 3000, Mettler Instrument Corporation, Greitensee, Switzerland) was used to study the effect of gallic acid and biochanin-A on the denaturation characteristics of BSA and soybean glycinin. The DSC was calibrated by using indium standards.

### 2.8.1. Sample preparation

Untreated proteins 20% were dissolved in phosphate buffer (pH 5, 7 and 9) ionic strength (0.01M). Soybean glycinin was dissolved in pH 7 and 9 buffer only. A total of 100  $\mu\text{L}$  solutions containing 20% proteins and 1% gallic acid and biochanin-A were prepared. The gallic acid and biochanin-A solutions were heated to 100°C in water bath for 7 min for solubilization then cooled to room temperature, before treating with protein solutions.

### 2.8.2. Sample Loading

For BSA 12  $\mu\text{L}$  and soybean glycinin 70  $\mu\text{L}$  of solution were placed in pre-weighed medium pressure DSC pans. The samples were scanned from 30°C to 90°C for BSA and 40°C to 120°C for soybean glycinin at a programmed heating rate of 10°C/min. For each run, a sample pan containing the buffer used for dissolving the proteins was used as control. After heating, the samples were allowed to cool to room temperature in the DSC and the heating cycle was repeated under the same experimental conditions. All DSC experiments were done in duplicate.

## 2.9. Fourier Transform Infrared Spectroscopy (FTIR)

An infrared spectrum was recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector.

### 2.9.1. Sample preparation

BSA and soybean glycinin (10% W/V) samples were dissolved in deuterium oxide containing 0.01 M phosphate buffer at pH 7 and 9. Gallic acid and biochanin-A were dissolved in phosphate buffer pH 7 and 9 (1 mg/50 $\mu\text{L}$ ); the phosphate buffer was initially freeze dried and water was replaced with deuterium oxide. Proteins and phenolic solutions were mixed together to have a final protein concentration of 10% containing 1% gallic acid and biochanin-A.

### 2.9.2. Sample loading

A total of 512 scans were averaged at 4  $\text{cm}^{-1}$  resolution. Wavelength accuracy was within  $\pm 1$  0.01  $\text{cm}^{-1}$ . The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, USA) the samples were held in an IR cell with a 50  $\mu\text{m}$  path length and CaF<sub>2</sub> windows. The temperature of the sample was regulated by placing the cell in a holder employing an Omega temperature controller (Omega Engineering, Laval, QC, Canada). The temperature was increased in 5°C increments, and the cell allowed equilibrating for 3 min prior to data acquisition. Deconvolution of the observed infrared spectra was performed using the Nicolet FTIR software, Omnic 1.2a. All FTIR experiments were done in duplicate.

## 3. Results and Discussion

### 3.1. Effect of pH on color changes of phenolic compounds

During the preparation of samples we observed color changes to solutions containing gallic acid at different pH and incubation temperatures. The protein-gallic acid solution at pH 7 incubated at 45°C changed color to light green, and dark green at pH 9 when incubated at 55°C. Friedman and Jurgens (2000) reported that resonance, hydrogen-bonding, hydrated structure and colors of phenolic compounds are strongly influenced by pH. No color changes were observed for solutions containing biochanin-A, suggesting that complex multi-ring aromatic structures are more stable to pH and temperature changes than mono-ring phenolic compounds. The above information is intended to aid in the reproducibility of our method.

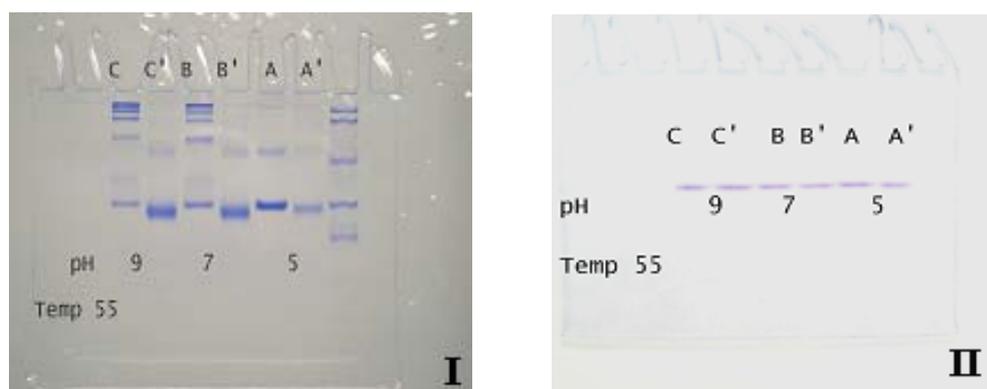
### 3.2. SDS-PAGE of proteins and phenolic acids

The SDS-PAGE results of BSA-gallic acid at 35°C, 45°C and 55°C and pH 3, 5, 7 and 9, showed no difference between the migration of BSA with and without gallic acid. On the basis of the information obtained in preliminary experiments pH 3.0 was excluded from the remaining experiments.

### 3.3. Native-PAGE of proteins and phenolic acids

Fig. 1 (I) shows the Native-PAGE electropherogram of BSA-gallic acid and BSA control. BSA-gallic acid showed relatively higher migration than BSA alone at all pHs and all temperatures (Fig. 1 (I), column A', B' and C'). The maximum difference in relative mobility (Retention Factor, Rf) was between BSA and BSA-gallic acid incubated at 55°C at pH 7 and 9. The difference in relative mobilities can explain the increase in net negative (–) charge on the native BSA molecule, as a result of gallic acid, as the migration of the protein in Native-PAGE is dependent on the charge of the protein molecule. BSA alone incubated at 55°C, at pH 7 and 9 (Fig. 1 (I), column A, B and C) showed loss of band intensities with the corresponding appearance of slower migration bands; these bands are very likely aggregation bands of BSA. BSA-gallic acid did not show the slower migration bands suggesting that the gallic acid inhibited the aggregation of BSA. BSA starts unfolding with subsequent denaturation and aggregation at 55°C; these changes are also affected by the pH of the medium. Some proteins are known to be relatively stable within a narrow pH range (4-7) however; exposure to pH outside this ranges often cause denaturation and subsequent aggregation of the proteins (Boye et al, 1996). The absence of aggregation bands of BSA-gallic acid is the indication of the protective effect against aggregation by the gallic acid. Inhibition of aggregation suggests that the hydroxyl groups of gallic acid can bind simultaneously at more than one site on the BSA molecule, which can prevent the unfolding of the BSA molecule. Fig. 1 (II) shows the electropherogram of BSA and BSA-biochanin-A; there was no marked difference between their relative mobilities. Suggesting minimal interaction between BSA and biochanin-A.

The electropherogram of soybean glycinin with and without gallic acid and biochanin-A is shown in Fig. 2 (I, II). The results show two discrete bands; one representing soybean glycinin and the other aggregate of soybean glycinin. There was no noticeable difference in the Rf value between glycinin, glycinin-gallic acid and glycinin-biochanin-A. The intensity of glycinin treated with gallic acid was less compared to glycinin alone (Fig. 2 (I), column E' and F'). The difference in the intensities can be a result of glycinin-gallic acid complexation, which could have resulted in precipitation of the glycinin. However, no difference was observed for the glycinin treated with biochanin-A (Fig. 2-II).



**Fig. 1.** Electropherogram (Native-PAGE) of BSA-gallic acid (I) and BSA-biochanin-A (II) at pH 5, 7 and 9 incubated at 55°C.

- A': BSA-gallic acid
- A: BSA
- B': BSA-gallic acid
- B: BSA
- C': BSA-gallic acid
- C: BSA



**Fig 2.** Electropherogram (Native-PAGE) of glycinin-gallic acid (I) and glycinin-biochanin-A (II) at pH 7 and 9 incubated at 55°C.

F': Glycinin-gallic acid  
 F: Glycinin  
 E': Glycinin-gallic acid  
 E: Glycinin

### 3.4. Thermal characteristics of BSA with phenolic acids

The effects of pH on the thermal characteristics of BSA with and without gallic acid are shown in Table 1. At pH 5 the denaturation temperature ( $T_d$ ) peak of BSA was at  $61.8 \pm 0.2^\circ\text{C}$ , which increased to  $62.0 \pm 0.1^\circ\text{C}$  for BSA-gallic acid. At pH 7,  $T_d$  for BSA was at  $58.2 \pm 0.1^\circ\text{C}$  and  $62.7 \pm 0.1^\circ\text{C}$  for BSA-gallic acid. At pH 9,  $T_d$  for BSA was at  $57.8 \pm 0.3^\circ\text{C}$  and  $62.6 \pm 0.2^\circ\text{C}$  for BSA-gallic acid. This data suggest that BSA-gallic acid showed greater thermal stability, than BSA alone at pH 7 and 9, but not at pH 5. The transition temperature of each domain in the order in which the domains unfold within a protein, has been reported to change with environment such as pH (Boye et al., 1996). The higher thermal denaturation temperature of BSA-gallic acid indicates higher thermal stability. This is consistent with the electrophoresis results, that gallic acid protected BSA against denaturation and aggregation at 55°C and pH 7 and 9. The  $T_d$  of BSA alone was lower than that of BSA-biochanin-A except at pH 5; suggesting the higher thermal stability of BSA-biochanin-A. At pH 9 the peak of denaturation shifted from  $57.8 \pm 0.3^\circ\text{C}$  to  $59.8 \pm 0.2^\circ\text{C}$ . Data suggest that BSA-gallic acid showed maximum stability at pH 9.

### 3.5. Thermal characteristics of soybean glycinin with phenolic acids

The thermal properties of soybean glycinin with and without gallic acid at pH 7 and 9 are shown in Table 1. The  $T_d$  for glycinin-gallic acid was higher  $92.5 \pm 0.1^\circ\text{C}$  to  $95.6 \pm 0.1^\circ\text{C}$  at pH 7, and  $92.4 \pm 0.3^\circ\text{C}$  to  $95.1 \pm 0.2^\circ\text{C}$  at pH 9, respectively, compared to glycinin alone. The higher denaturation temperature reflects higher thermal stability of glycinin-gallic acid, compared to glycinin alone. The  $T_d$  of soybean glycinin and glycinin-biochanin-A were similar  $92.5 \pm 0.1^\circ\text{C}$  and  $92.7 \pm 0.1^\circ\text{C}$  at pH 7 and  $92.4 \pm 0.2^\circ\text{C}$  to  $93.0 \pm 0.2^\circ\text{C}$  at pH 9, respectively.

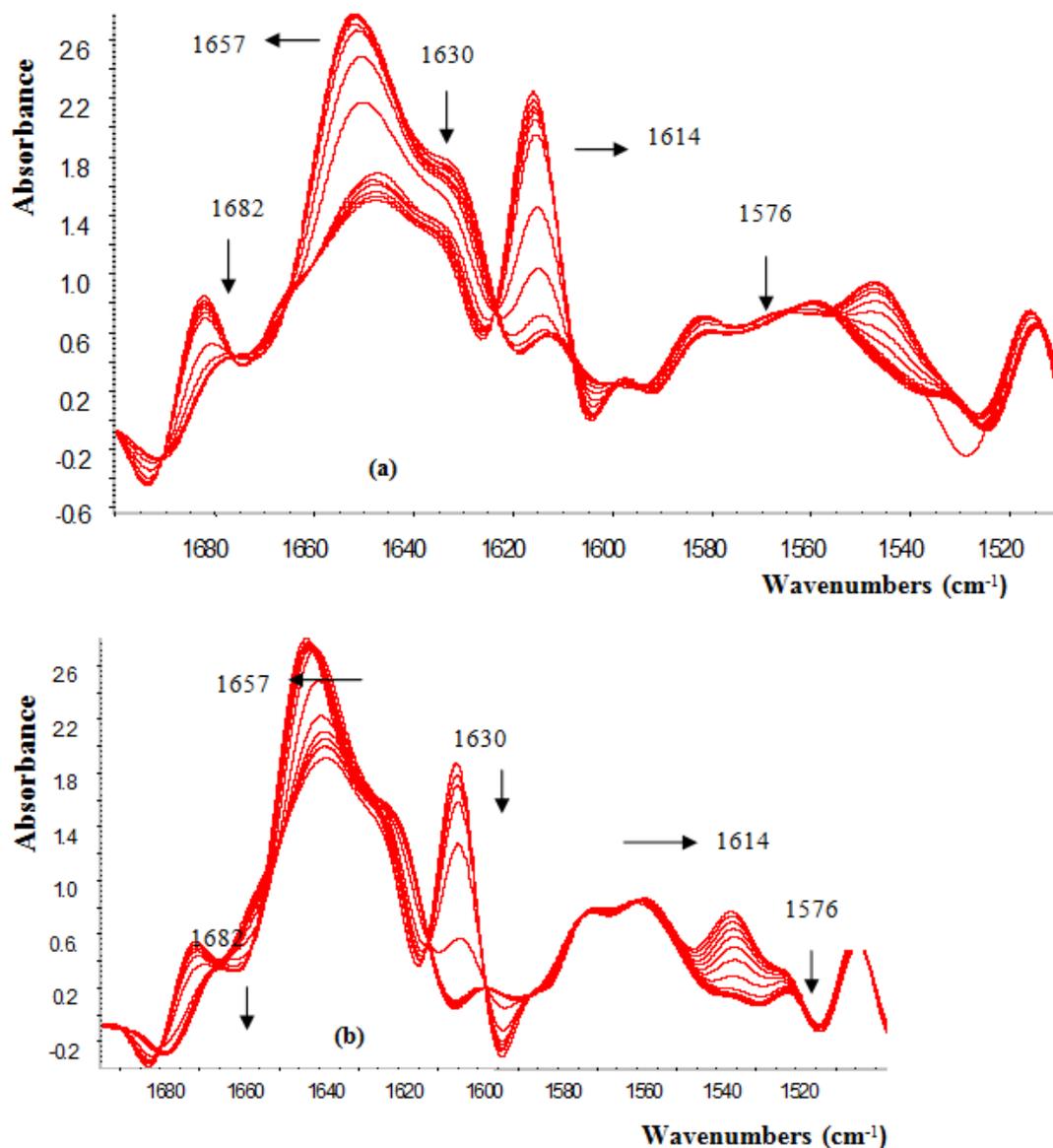
Both  $T_d$  and  $\Delta H$  of soybean glycinin increased in the presence of gallic acid indicating conformational structural changes of soybean glycinin, possibly resulting in the formation of protein-phenolic complexes through hydrogen bonding and hydrophobic interaction. These conformational changes were not observed in the presence of biochanin-A.

**Table 1.** Effects of pH on thermal characteristics of BSA and glycinin with and without gallic acid and biochanin-A.

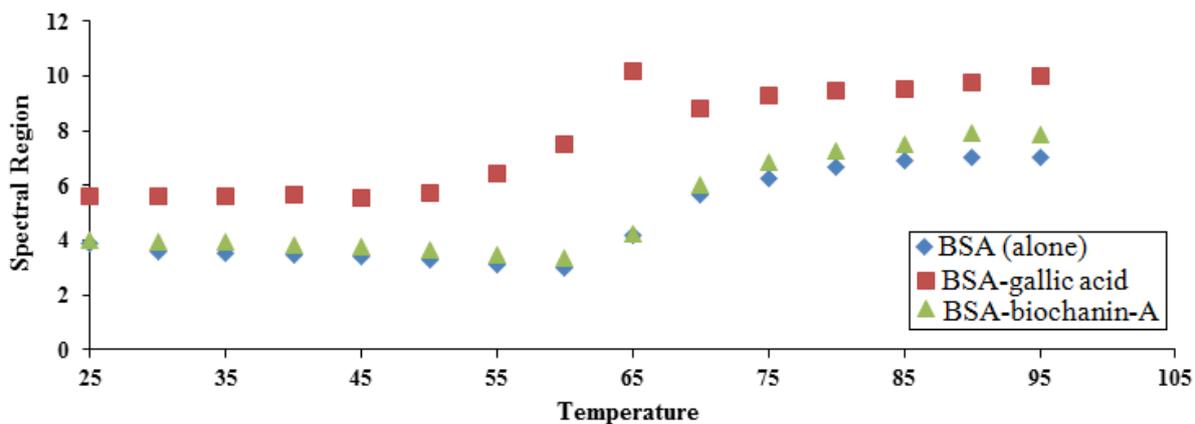
Treatment (pH)	Denaturation Temperature (Td °C)	Enthalpy ( $\Delta H$ )
BSA	pH 5	61.8 $\pm$ 0.2°C
	pH 7	58.2 $\pm$ 0.1°C
	pH 9	57.8 $\pm$ 0.3°C
BSA-gallic acid	pH 5	62.0 $\pm$ 0.1°C
	pH 7	62.7 $\pm$ 0.1°C
	pH 9	62.6 $\pm$ 0.2°C
BSA-biochanin-A	pH 5	60.3 $\pm$ 0.1°C
	pH 7	59.8 $\pm$ 0.1°C
	pH 9	59.8 $\pm$ 0.2°C
Glycinin	pH 7	92.5 $\pm$ 0.1°C
	pH 9	92.4 $\pm$ 0.3°C
Glycinin-gallic acid	pH 7	95.6 $\pm$ 0.1°C
	pH 9	95.1 $\pm$ 0.2°C
Glycinin-biochanin-A	pH 7	92.7 $\pm$ 0.1°C
	pH 9	93.0 $\pm$ 0.2°C

### 3.6. Effect on secondary structure of BSA and phenolic acids

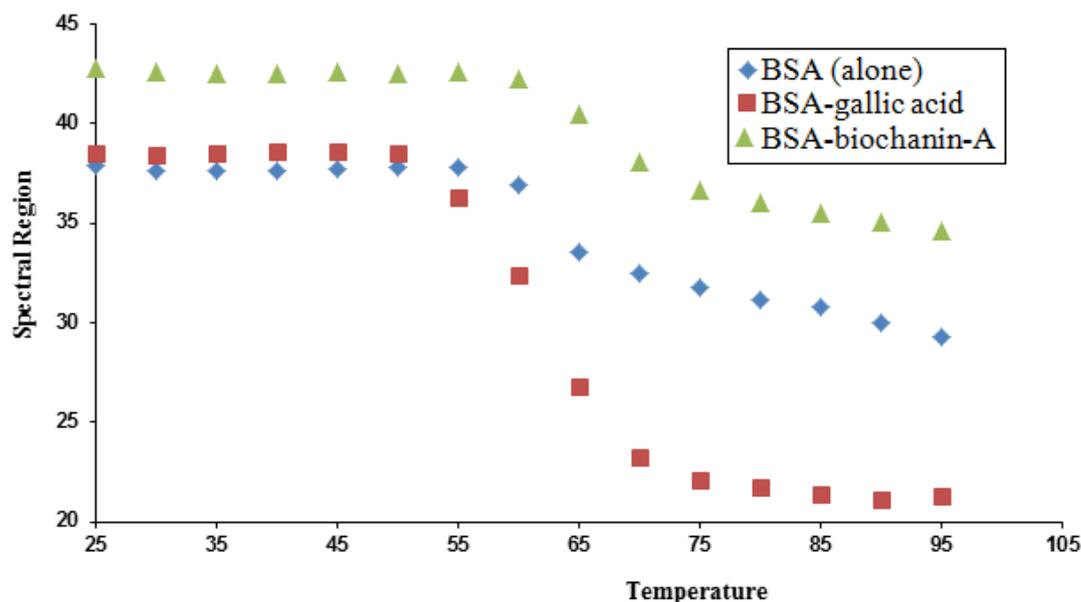
The deconvolved infrared spectra of BSA (10%) recorded immediately after dissolving BSA-gallic acid and BSA-biochanin-A in deuterated phosphate buffer (pH 7), heated from 25°C to 95°C are shown in Fig. 3. An intense peak at 1657  $\text{cm}^{-1}$  in the infrared spectrum of BSA is attributed to  $\alpha$ -helical conformation, a shoulder at 1630  $\text{cm}^{-1}$  is attributed to  $\beta$ -sheets, whereas the 1614 and 1682  $\text{cm}^{-1}$  bands have been attributed to intermolecular hydrogen bonded  $\beta$ -sheet structure (Clark et al, 1981). To investigate the effect of gallic acid and biochanin-A on the intermolecular hydrogen bonded  $\beta$ -sheet structure of BSA, the integrated intensity in the region between 1682-1674  $\text{cm}^{-1}$  was plotted as a function of increasing temperature (Fig. 4). The result shows that, starting at 55°C at pH 7, there was an increase in the band intensity. However biochanin-A had no effect on the thermal denaturation of BSA. The integrated intensity in  $\alpha$ -helical domains of BSA, the region between 1657-1643  $\text{cm}^{-1}$ , showed faster rate of decrease in the presence of gallic acid at pH 9 (Fig. 5). Due to the complexity of the soybean glycinin the spectra could not be acquired on FTIR, consequently the effect of gallic acid and biochanin-A on the secondary structure could not be investigated.



**Fig. 3.** Deconvoluted infrared spectra of BSA (a) BSA-gallic acid (b), and BSA-biochanin A at pH 7.



**Fig. 4.** Plots drawn between the spectral region 1682-1674  $\text{cm}^{-1}$  versus temperature  $^{\circ}\text{C}$  of BSA with and without gallic acid and biochanin-A at pH 7.



**Fig. 5.** Plots drawn between the spectral region  $1657\text{-}1643\text{ cm}^{-1}$  versus temperature  $^{\circ}\text{C}$  of BSA with and without gallic acid and biochanin-A at pH 9.

#### 4. Conclusion

Results from this research indicate that single ring phenolic acid have a much higher binding tendency to proteins compared to multi aromatic ring isoflavone. This can be demonstrated by Native-PAGE and DSC but not FTIR. Both pH and the temperature have shown impact on these interactions. The BSA-gallic acid showed difference in relative mobility when subjected to Native-PAGE. Further, gallic acid also prevented protein aggregation at higher incubation temperatures. However the results were not similar when BSA was treated with isoflavone, biochanin-A. Thermal analysis using DSC confirmed higher thermal stability of the proteins treated with phenolic compounds. The FTIR spectroscopy showed some differences in the secondary structure of the protein-phenolic complexes compared to protein alone.

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