Preparation and Evaluation of Tara-modified Proteins*

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Abstract

Quebracho, a vegetable tannin, can be used to modify gelatin to produce a product that has been applied effectively as a filler in leather processing, as described in our previous report. In this ongoing study, another vegetable tannin, tara, is examined for its possible application in protein modification. The tara tanning substance is a gallotannin, comprised mainly of gallic acid, and is extracted from the pods of the small tara tree (*caesalpinia spinosa*); it is a native plant species of Peru. Advantages for using tara are that it not only gives an almost colorless product, which would be desirable in production of light colored leather, it also imparts light-fastness to the resultant leather and is a truly sustainable resource. We examined the possibility of using both tara and its main component, the polyphenolic gallic acid, as crosslinking agents for gelatin and whey protein concentrate (WPC). Protein modification parameters were developed and the results of product characterization using physicochemical analyses showed that products had improved physical properties. The molecular weight distribution indicated that the bands indicative of gelatin and whey had been altered suggesting higher molecular weight moieties. Thus, by-products from the leather and dairy industry, modified by using a sustainable resource, have the potential to be used in leather processing, specifically as fillers.

Resumen

Quebracho, un tanino vegetal, puede utilizarse para modificar gelatina para producir un producto que se ha aplicado eficazmente como rellenante en el tratamiento del cuero, como se describe en nuestro informe anterior. En este estudio en curso, otro tanino vegetal, tara, es examinado para su posible aplicación en la modificación de proteínas. La sustancia curtiente de la tara es una galotanino, compuesto principalmente de ácido gálico, y se extrae de las vainas del pequeño árbol de tara (Caesalpinia spinosa), una especie de planta nativa del Perú. Las ventajas para el uso de tara son que no sólo da un producto casi incoloro, que sería deseable en la producción de cueros de color claro, sino que también imparte resistencia a la luz a la piel resultante y es un recurso verdaderamente sostenible. Se examinó la posibilidad de utilizar tanto tara y su componente principal, el ácido gálico polifenólico, como agente de reticulación para el concentrado de proteína de gelatina y suero (WPC). Los parámetros de modificación de proteínas se desarrollaron y los resultados de la caracterización del producto usando análisis fisicoquímicos mostraron que los productos han mejorado las propiedades físicas. La distribución de peso molecular indica que las bandas indicativas de gelatina y suero han sido alterados sugiriendo fracciones con un mayor peso molecular. Por lo tanto, los subproductos de la industria del cuero y productos lácteos, modificados mediante el uso de un recurso sostenible, tienen el potencial de ser utilizados en el tratamiento del cuero, específicamente como agentes de relleno.

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INTRODUCTION

The polyphenolic acids in vegetable tannins have been investigated at length for their ability to modify gelatin.[1-6] They are known to react under oxidizing conditions with side chain amino groups of peptides, leading to formation of cross-links in proteins.[1] Kosaraju, et al.[6] demonstrated that gelatin could be reacted with caffeic acid which resulted in a cross-linked structure with a rigid protein matrix that was stable even under boiling, and that different degrees of crosslinking could be realized by altering parameters such as pH, time and concentration of caffeic acid. In addition, it has been reported that some vegetable tannins themselves could be applied to gelatin to give products with interesting physical properties.[7] Whey also had been examined for its reactivity with polyphenolics. Rawel, et al. reported that β-lactoglobulin, one of the components of whey, reacts with polyphenolic acids at pH 9.0 and a change is noted in the molecular weight distribution.[8]

We recently demonstrated that gelatin could be modified with the vegetable tannin quebracho to produce a product that could be used effectively as a filler in leather processing.[9,10] In this continuing study, the vegetable tannin tara and its main component, the polyphenolic, gallic acid which is known to react with gelatin,[11] are examined for their possible application in modification of proteins. The tara tanning agent is extracted from the fruit husks of the small tara tree (*cesalpina spinosa*). The plants grow predominantly wild in Peru, in the mild climate of the valleys of the Andes.[12] Tara is a hydrolysable tannin with mixtures of polygalloyl glucoses and/or poly-galloyl quinic acid derivatives containing between 3 to 12 gallic acid residues per molecule. Hydrolyzable tannins are hydrolyzed by weak acids or weak bases to produce carbohydrate and phenolic acids. Tara is a gallotannin composed of depsidically connected units of gallic acid surrounding a polyolic core.[13] Among the advantages for using tara are that it gives an almost colorless product, which would be desirable in production of light colored leather and it imparts light-fastness to leather. Furthermore, as described in a recent paper by Castell et al, tara used in tannage for producing wet white, is a truly sustainable resource.[14]

Conditions (pH, time temperature and concentration) for tara modification of gelatin and WPC will be determined. Products will be characterized with respect to physical properties and molecular weight distribution. Gallic acid which is the main component of tara will also be examined for its capacity to modify gelatin and WPC. Properties of the various products will be discussed and suggestions made for their potential uses in leather processing, e.g. fillers.

EXPERIMENTAL

Material

Two samples of commercial Type B gelatin from bovine skin, characterized in this laboratory as 95g and 175g Bloom, were obtained from Sigma-Aldrich Corp. (St. Louis, MO) and Fisher Scientific (Fairlawn, NJ), respectively. Whey protein concentrate containing 80% protein, (Hilmar™ 8000) was generously supplied by Hilmar Ingredients (Hilmar, CA). Tara was obtained from Hermann Oak Leather Company (St. Louis, MO). Gallic acid was obtained from Sigma-Aldrich Corp. (St. Louis, MO). All other chemicals were analytical grade and used as received.

Preparation of Tara and Gallic Acid-modified Gelatin and WPC Products

Gelatin (95g and 175g Bloom) and WPC samples, 5 g of each, were suspended in water (40 mL) held for 2 h at room temperature (25-28°C) and then stored overnight at 4°C. They were placed in a bath at 65 ºC until dissolved. Control samples to which no tannin was added, were run to monitor changes in physical properties. The pH was adjusted to 4.0-10.0 with 1 N HCl or 1 N NaOH. Tara and gallic acid (calculated to be 0 to 7.5% based on weight of total protein) solutions were prepared in 10 mL of water. The tara solutions were first heated to dissolve the product, centrifuged, and the supernatant was added with stirring to the protein solutions to give final protein concentration of 10% w/v. The gallic acid solutions were also added to their respective protein solutions to give the same concentration (10% w/v). Aliquots (10 mL) of all the reaction mixtures were added to test tubes for melting point determination and 30-mL aliquot was poured into appropriate containers (39-mm diameter jar) for determining gel strength. The samples were warmed to 45°C in a shaker bath and the reaction was carried out for 4 h. The samples were cooled to room temperature and then chilled for 17 h at 10 ºC in a constant temperature bath. If appropriate, physical analyses (gel strength, melting point and viscosity) were run on these samples. Aliquots of the samples were lyophilized and molecular weight distribution was determined. Sodium azide (70 μL of 1% solution) was added to the remaining treatment solution as a preservative and the samples were stored at 4°C.

ANALYSES

Physical Properties and Molecular Weight Distribution

Gel strength, melting point, and viscosity of the tara and gallic acid-treated proteinaceous solutions were determined as described in previous publications.[15] Protein molecular weights were estimated as described previously.[16] In summary, SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) was run using precast 4-15 percent gradient gels. A broad range SDS-Standard (BRS) calibration standard (Bio-Rad, Hercules, CA), which contains a mixture of nine proteins ranging in size from 6,500 to 200,000 Daltons, was used. Samples of lyophilized protein were dissolved in sample buffer (10 mM Tris-HCl at pH 8.0 containing 1 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and were then heated at 40°C for 4 h. Separation was achieved using a Phast-Gel System (Pharmacia Biotech Inc., Piscataway, NJ). Gels were stained with Coomassie Blue (Pharmacia).

Hydrothermal Stability

Hydrothermal stability of tara and gallic acid-modified gelatin and WPC were determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.[17] In preparation for DSC experiments, unmodified and modified-gelatin and WPC (100-150 mg) samples were weighed into ampoules and a small amount of distilled water (500 μL) was added; the ampoules were sealed and placed in the calorimeter. The calorimeter was programmed to record heat flow as μcal/°C while the temperature was increased from 10°C to 180°C at 1.0°C/min with an equilibration period of 600 s at the start. The temperature at the peak of the calorimetry trace, $T_{p,}$ was considered to be an apparent melting temperature.

SEM Imaging

Gelatin gel samples (5- x 10- mm) were immersed in 20 mL aliquots of a fixative solution containing 2.5% glutaraldehyde-0.1 M imidazole-HCl (pH 7.2) in sealed vials for about 6 h, at room temperature. Subsequently, the fixative solution was decanted and exchanged with 0.1M imidazole buffer, and the samples were dehydrated by exchange with 20-mL volumes of graded ethanol solutions, 50%, 80%, and absolute ethanol. Three changes were carried out at each concentration for 60 min. Finally the gel samples were frozen in liquid nitrogen and fractured with cold scalpel blades, and fragmental samples were critical point dried from liquid CO_2 in a DCP-1 Critical Point Dryer (Denton Vacuum, Inc., Cherry Hill, NJ). The samples were mounted on specimen stubs using glue (Duco Cement, ITW Performance Polymers, Riviera, FL) and the edges painted with colloidal silver adhesive (Electron Microscopy Sciences, Hatfield, PA), then sputter coated with a thin layer of gold using a Scancoat Six Sputter Coater (BOC Edwards, Wilmington, MA). Digital images of topographical features of the gel samples were collected using a Quanta 200 FEG environmental scanning electron microscope (FEI Co., Inc., Hillsboro, OR) operated in the high vacuum/secondary electron imaging mode at an accelerating voltage of 10 kV and instrumental magnifications of 50x, 500x and 1,000x.

RESULTS AND DISCUSSION

Preparation and Characterization of Tara-modified Gelatin

Gelatins of various Bloom strengths (95 g, trial A and 175 g, trials B-D) were modified at pH 4.0 to 10.0 with 2.5 and 3% tara as described in the Experimental section, and the physicochemical properties were examined. Because of a preponderance of insolubles in the tara product, the tara solutions were first centrifuged and the resulting supernatant was added to the gelatin solutions; reaction proceeded at 45°C for 4 h. Physical properties were examined and as seen in Figure 1a the gel strengths of both Bloom samples increased up to pH 6.0, but decreased after that. At the same time the melting points slowly increased as the pH was raised to pH 10.0 (Figure 1b) as did the viscosity (Figure 1c). The physical properties of unmodified gelatin control samples, when subjected to reaction in the pH 4-10 range, changed little, an observation reported in prior publications.[9,18]

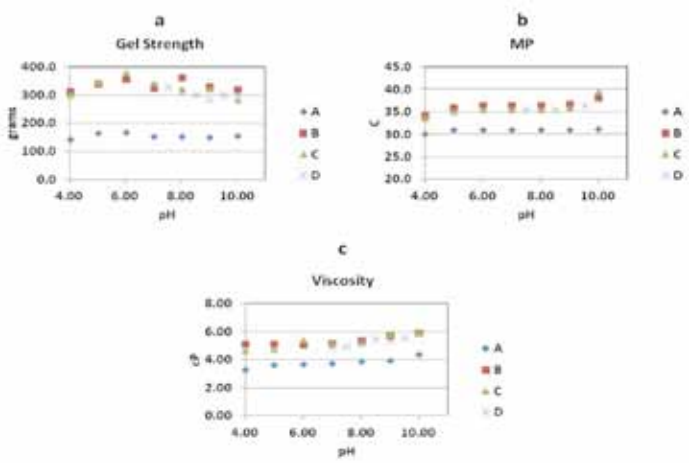


Figure 1. 95 (A) and 175 (B) Bloom gelatin (10% w/v) treated with 2.5% tara, pH 4.0-10.0; 175 Bloom gelatin (10% w/v) (C and D) treated with 3% tara, pH 4-10.0; all at 45°C for 4 h; (a) gel strength; (b) melting point; (c) viscosity at 60°C.

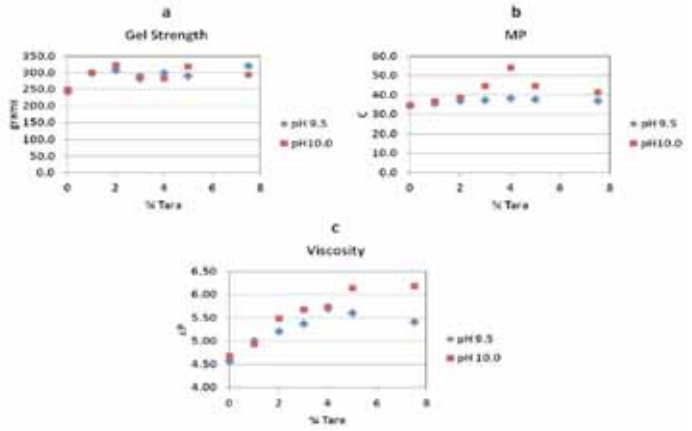


Figure 2. 175 Bloom gelatin (10% w/v) treated with 0-7.5% tara at pH 9.5 and 10.0, 45°C for 4 h (a) gel strength; (b) melting point; (c) viscosity at 60°C.

With the pH remaining constant at 9.5 and 10.0 (Figure 2) and when the concentration of tara was increased from 0 to 7.5%, the gel strength (Figure 2a) increased with increasing concentration of tara up to 4%, where there was a decrease and then there was an increase above 5%; the melting point (Figure 2b) followed the same trend, whereas the viscosity (Figure 2c) rose with increasing tara concentration and then appeared to level off above 4%. When the molecular weight was examined at both pH 9.5 and 10.0 (Figure 3), the bands for gelatin become less distinct at 3-4% concentrations (at both pH 9.5 and 10.0) and are possibly indicating that the reaction had reached its maximum and further addition of tara inhibited the reaction.

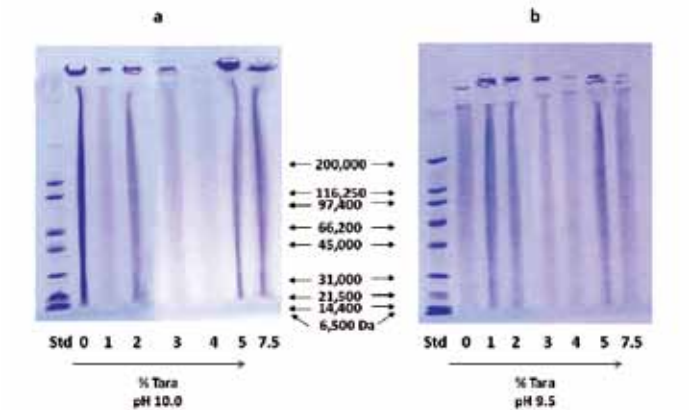
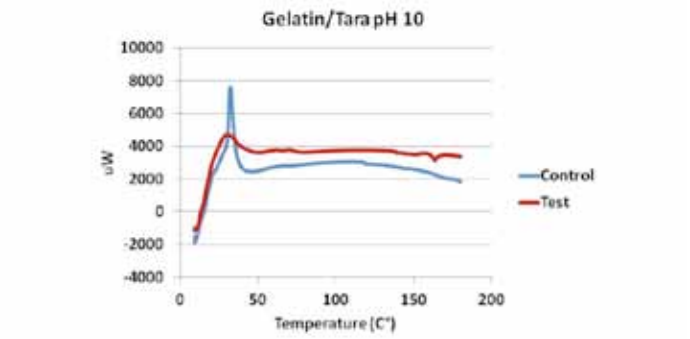


Figure 3. SDS-PAGE of 175 Bloom gelatin (10% w/v), treated with 0-7.5% tara at pH 10.0 (a) and with 0-7.5% tara at pH 9.5, (b), 45°C for 4 h; molecular weights are shown in Da.

When the 4% tara-modified gelatin (pH 10.0) and its control were examined by DSC (Figure 4), the endothermic or melting point of unmodified gelatin was about 31.9°C and the peak was sharp; the modified gelatin sample gave a broader, less distinct peak at about 31.4°C and an exothermic peak appeared at about 163.1°C.



Sample	Endothermic Peak (°C)	Exothermic Peak (°C)
10% Gelatin/0% tara (Control)	31.9	-
10% Gelatin/4% tara (Test)	31.4	163.1

Figure 4. DSC analysis of 175 Bloom gelatin (10% w/v) treated with 0% tara at pH 10 (Control), and with 4% tara at pH 10 (Test), 45°C for 4h; table indicates melting (endothermic) and exothermic temperature peaks.

Preparation and Characterization of Gallic Acid-modified Gelatin

It has been suggested in the literature[11] that gallic acid modification of gelatin could affect the physicochemical properties of the gelatin products. We designed several experiments in which we first modified gelatin at pH 9.0 using 0-5% gallic acid. At the 1% concentration (Figure 5a, b, and c) there was an increase in gel strength, melting point, and viscosity, and these properties gradually decreased with increasing gallic acid concentrations.

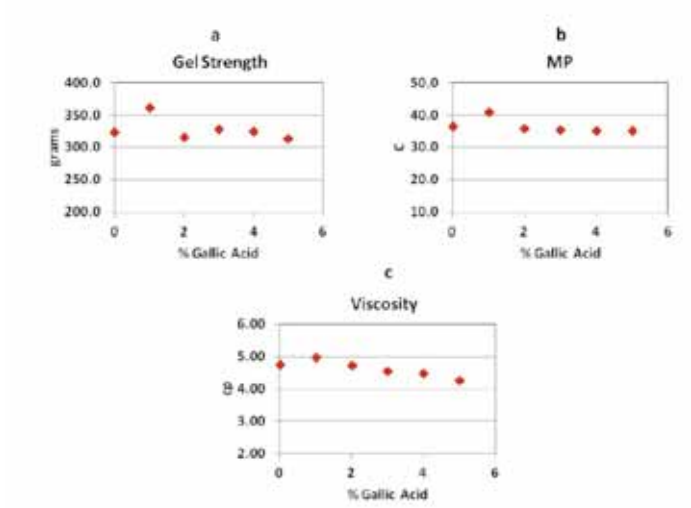


Figure 5. 175 Bloom gelatin (10% w/v) treated with 0-5% gallic acid at pH 9.0, 45°C for 4 h (a) gel strength; (b) melting point; (c) viscosity at 60°C.

Molecular weight distribution (Figure 6a) indicates that even though the physical properties are not showing change, the bands indicative of gelatin are slowly disappearing with the higher concentrations.

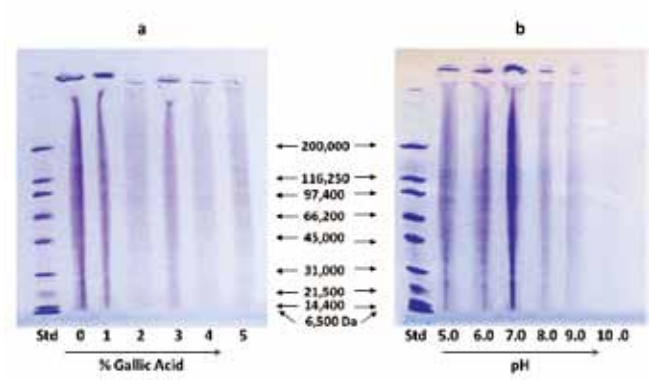
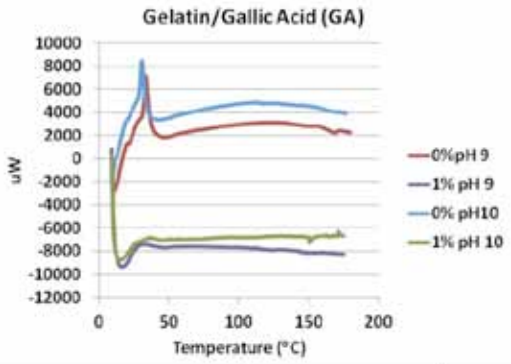


Figure 6. SDS-PAGE of 175 Bloom gelatin (10% w/v), treated with 0-5% gallic acid at pH 9.0 (a), and with 1% gallic acid at pH 5.0-10.0, (b), 45°C for 4 h; molecular weights are shown in Da.

DSC (Figure 7) shows that the exothermic melting peak of the modified 1% gelatin sample has diminished and has become broader; no exothermic peak in this temperature range is observed for the modified sample.

Because of lack of correlation between the physical properties and molecular weight distribution, we decided to make a further change in that the 1% gallic acid concentration was examined at pH 5-10. We had initially decided on pH 9.0 because of experience with tara (being composed mainly of gallic acid). We found (Figure 8a, b, c) that at pH 10.0, the gel strength drops dramatically, the sample will not melt and in turn no viscosity could be read. When molecular weight distribution studies were carried out (Figure 6b), the bands indicative of gelatin have almost disappeared; this correlates

with the physical property data, particularly with respect to gel strength falling off so dramatically and is possibly indicative of so many crosslinks being introduced the gel strength collapses. DSC studies (Figure 7) indicate that the endothermic peak for the modified sample (at pH 10.0) has been diminished and is quite broad; an exothermic peak is also seen at temperature of 152.0°C.



Sample	Endothermic Peak (°C)	Exothermic Peak (°C)
10% Gel/0% GA (Control) pH 9	32.6	
10% Gel/1% GA (Test) pH 9	31.3	
10% Gel/0% GA (Control) pH 10	31.8	-
10% Gel/1% GA (Test) pH 10	34.2	152.0

Figure 7. DSC analysis of 175 Bloom gelatin (10% w/v), treated with 0% and 1% gallic acid (GA) at pH 9.0 and at pH 10.0, 45°C for 4h; table indicates melting (endothermic) and exothermic temperature peaks.

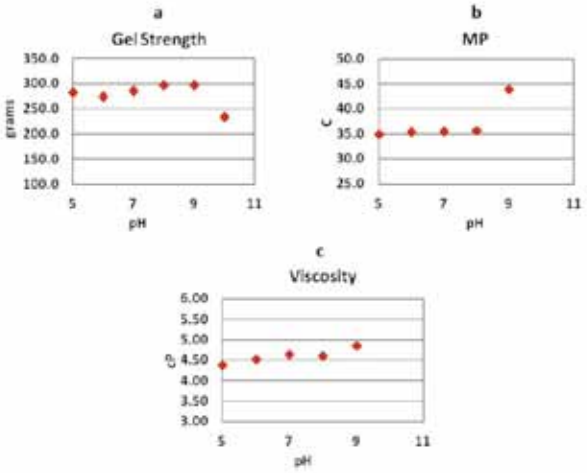


Figure 8. 175 Bloom gelatin (10% w/v), treated with 1% gallic acid at pH 5-10, 45°C for 4 h (a) gel strength; (b) melting point; (c) viscosity at 60°C.

SEM imaging of the tara and gallic acid treated gelatin indicated a difference in structure between modified and unmodified samples (Figure 9). When these samples were being prepared, one of the steps is fracturing of the dried gels and there was difficulty in fracturing the test samples (both tara treated and gallic acid treated) in that the samples had a tendency to shatter.

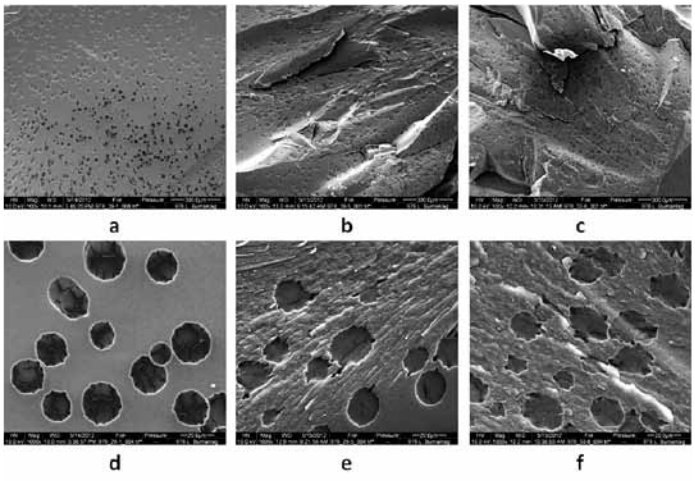


Figure 9. SEM micrographs of 175 Bloom gelatin, untreated control, 10% w/v (a), tara-treated gelatin,10% w/v, 4% tara, pH 10.0 (b), gallic acid-treated gelatin, 10% w/v, 1% gallic acid, pH 10.0 (c), at 100X(— = 500μm); untreated control 10% w/v (d), tara-treated gelatin (10% w/v, 4% tara, pH 10.0 (e), gallic acid-treated gelatin 10% w/v, 1% gallic acid, pH 10.0 (f), at 1000X (— = 20μm); all treated at 45°C for 4 h.

Images shown in Figure 9a-f are reflecting this in that the unmodified control sample, in both the 100x and 1000x magnification, is showing a smooth type surface structure whereas the tara and gallic acid-modified gelatins are quite rough and reflect the splintering and shattering of the samples.

Concentration of tara and gallic acid and pH of the reaction affect physicochemical properties of gelatin. The molecular weight distribution of tara-modified gelatin (pH 9.0-10.0) agrees with physical properties, however, the gels for gelatin-gallic acid (pH 9.0) do not, in that the bands indicative for gelatin continually disappear as the concentration of gallic acid is increased. By increasing the pH of the reaction to 10.0, a dramatic change in physical properties is seen and correlates with molecular weight distribution. The data suggest that 4% tara at pH 9.0-10.0 and 1% gallic acid at pH 9.5-10.0 will give optimal physical properties when making, for example, products for the filling of leather.

Characterization of Tara-and Gallic Acid-modified WPC

Rawel, et al[8] suggested that one of the components of whey (β-lactoglobulin) should react with polyphenolic acids at pH 9.0 and a change should be noted in the molecular weight distribution. Experiments were run in which various concentrations of tara and gallic acid were reacted with WPC at pH 9.0. Initially, the viscosity, in both the tara and gallic acid treated WPC solutions increased slightly from that of an untreated control sample and then slowly decreased (Figure 10a).

However when the molecular weight distribution was examined it appeared (Figure 10b and c) that there is an indication that a reaction is taking place. In WPC there are

bands on gel for α-lactalbumin, MW=14700, β-lactoglobulin, MW=18300, and β-lactoglobulin dimer;[19] the latter two are diminished in the tara treated samples. In the gallic acid treated WPC samples the changes are less noticeable. DSC analyses for tara samples (Figure 11a) (control, a = 1% and b = 5% tara) show that a peak for the control at approximately 25°C is not seen in the treated samples. DSC analyses for gallic acid-treated WPC samples (Figure 11b) again show that the peak for the control (at about 25°C) has disappeared, but in the area of about 38°C and 75°C a broad peak is seen in both test samples and is more definitive in the Test B sample which had the higher concentration of gallic acid (5%) applied.

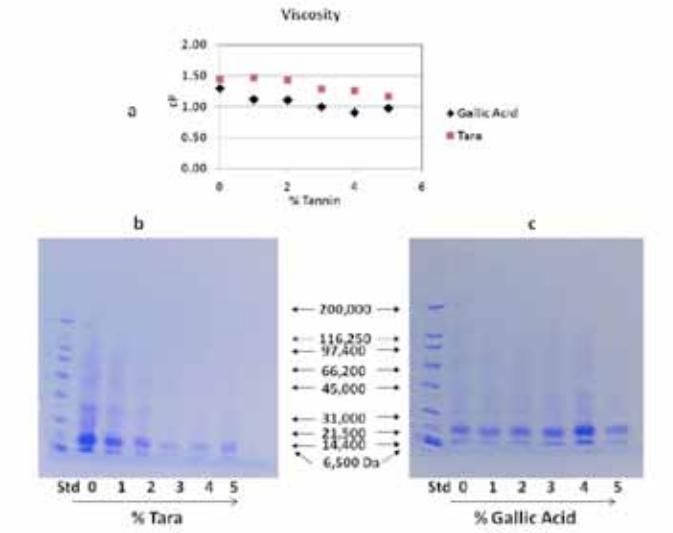


Figure 10. Viscosity at 60°C (a) and SDS-PAGE of WPC (10% w/v), treated with 0-5% tara (b) and 0-5% gallic acid (c) at pH 9.0, 45°C for 4 h; molecular weights are shown in Da.

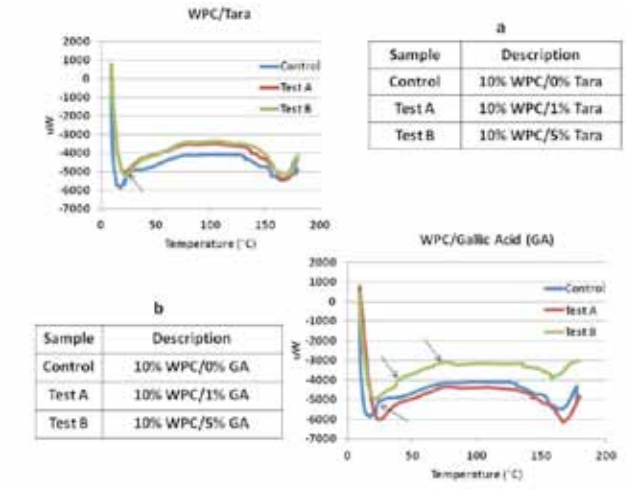


Figure 11. DSC analysis of WPC (10% w/v), treated with 0, 1, and 5% tara (a) and 0, 1, and 5% gallic acid (GA) (b), at pH 9.0, 45°C for 4h.

WPC has been treated with both tara and gallic acid, and SDS-PAGE analysis of tara treated samples suggest, as has been reported in the literature, that β-lactoglobulin and its dimer have reacted with the phenolics; with respect to gallic acid, it appears that the dimer has been most affected. DSC analyses of tara-and gallic acid-modified WPC, indicated that the profile of WPC changes in both reactions after modification.

CONCLUSIONS

Tara and gallic acid can react with gelatin to give products with improved physicochemical properties. SDS-PAGE analyses of both tara-and gallic acid-modified gelatin, suggest the formation of higher molecular weight moieties. WPC has been treated with both tara and gallic acid, and SDS-PAGE analyses of tara samples suggest, as has been reported in the literature, that β-lactoglobulin and its dimer have reacted with the phenolics. With respect to gallic acid, the dimer seems to be most affected. DSC analyses of tara-and gallic-acid-modified gelatin and WPC, indicated that the profiles of the proteins change after modification.

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