

# BIOPOLYMERS PRODUCED FROM GELATIN AND CHITOSAN USING POLYPHENOLS\*

by

M.M. TAYLOR,† L.P. BUMANLAG, E.M. BROWN AND CHENG-KUNG LIU  
*United States Department of Agriculture, Agricultural Research Service  
 Eastern Regional Research Center  
 600 E. MERMAID LANE, WYNDMOOR, PA, 19038*

## ABSTRACT

Chitin, and its derivative chitosan, is an abundant waste product derived from crustaceans (e.g. crab). It has unique properties, which enable its use in, but not limited to, cosmetic, medical and food applications. Chitosan has recently been studied, in conjunction with other waste carbohydrates and proteins, for the purpose of making biopolymer products with unique functional properties. Furthermore use of renewable polyphenols to assist in these reactions is a topic of growing interest. In prior research, we investigated the use of polyphenols, specifically gallic acid and the vegetable tannins quebracho and tara, to modify gelatin. We improved the physical properties of gelatin and were able to demonstrate that these products could be used effectively as fillers. At present, gelatin is scarce and becoming increasingly more expensive so we produced biopolymers from modification of gelatin and whey using the vegetable tannin tara, and made products to augment the gelatin; we further demonstrated that the resulting product could be used as a filler for leather. This present study investigated the preparation of a biopolymer using gelatin and chitosan, modified with the vegetable tannin tara, to make products that could be used either in leather making process, e.g. as fillers or coatings or could have other potential uses such as in film-making or as flocculants. Optimal conditions necessary for polyphenols to react with gelatin and chitosan were determined and physical properties showed that unique products were produced. The gels were examined for thermal stability and for fluorescence. We thus demonstrated that gelatin/chitosan/tara products are feasible and that gelatin products could be supplemented using an inexpensive abundant waste product, chitosan.

## INTRODUCTION

Gelatins are increasingly in short supply<sup>1</sup> and the costs are subsequently escalating. Replacements for gelatin are being investigated, primarily with respect to supplementing some of the gelatin with another substrate. Several researchers have established the feasibility of producing biopolymers from the reaction of polyphenols with gelatin in combination with other proteins or with carbohydrates. For example, Zhang et al.<sup>2</sup> used tannic acid to produce gelatin-gum arabic coacervate microspheres, Strauss and Gibson<sup>3</sup> and Mathew and Abraham<sup>4</sup> showed that biopolymers could be produced by reaction of polyphenols with gelatin and pectins as well as with starch and chitosan. As described, these combinations would take advantage of the unique properties of both classes of waste products and simultaneously generate products with improved functional properties.

In prior research, we have investigated the use of polyphenols, specifically gallic acid and the vegetable tannins quebracho and tara, to modify gelatin.<sup>5-8</sup> We successfully improved the physical properties of gelatin and were able to demonstrate that these products could be used effectively as fillers.<sup>6,8</sup> We also investigated the modification of whey protein concentrate (WPC) using the above referenced polyphenols and found that gallic acid and tara would be effective in modification of WPC. Based on this reported research, we recently produced biopolymers from modification of gelatin and whey using the vegetable tannin tara.<sup>9</sup> We further demonstrated that the resulting product could be used effectively as a filler for leather.<sup>10</sup>

\*Presented in part at the 110th annual American Leather Chemists Association Meeting, June 18-20, 2014, Gideon Putnam Resort, Saratoga Springs, NY

†Corresponding author e-mail: maryann.taylor@ars.usda.gov

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

Manuscript received June 17, 2015, accepted for publication July 21, 2015.

Encouraged by our success with the WPC protein as a partial replacement for gelatin, and the reported ability of chitosan to react with polyphenols, we designed experiments using chitosan and gelatin, in conjunction with tara, to see if we could make a biopolymer product that would have unique functional properties in that they could be used in films, coatings, flocculants or as fillers. Chitin is an abundant waste product; it is a natural polysaccharide derived from the exoskeleton of crustaceans (e.g. crab and shrimp). Chitosan, derived by deacetylation of chitin, has many uses, for example in water treatment,<sup>11-12</sup> as an antimicrobial<sup>13-16</sup>, as an antioxidant,<sup>17-18</sup> in making of biomedical devices,<sup>19</sup> and based on its ability to make good films, it could be used in packaging, and many other applications.<sup>4, 14-17, 20-26</sup>

The reaction of chitosan with polyphenols has been reported in the literature. The improvement in antimicrobial, mechanical, physical and structural properties of chitosan films that were incorporated with gallic acid are outlined by Sun et al.<sup>16</sup> Siripatrawan and Harte produced films from chitosan incorporated with green tea extract resulting in improved mechanical properties and improved antioxidant activity.<sup>17</sup> Chitosan films incorporated with tea polyphenol were developed as packaging material for extending the shelf life of pork meat patties (Qin et al.<sup>20</sup>). Wang et al, also described the uses of films prepared with chitosan and tea polyphenols.<sup>21</sup> Rivero et al, discussed crosslinking capacity of tannic acid in plasticized chitosan films with emphasis on physicochemical, barrier and mechanical properties.<sup>23</sup> The characterization of ferulic acid incorporated starch–chitosan blend films for possible edible films or coatings were reported by Mathew and Abraham.<sup>4</sup> With respect to chitosan/gelatin biopolymers, Rocasalbas et al, confirmed the formation of bioactive chitosan/gelatin hydrogel stabilized with plant polyphenols for purpose of wound treatment also because of their antibacterial properties.<sup>27</sup> Gómez-Estaca et al, described preparation of biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation.<sup>14</sup> The effects of gelatin origin, bovine-hide and tuna-skin, on the properties of compound gelatin–chitosan films were outlined by Gómez-Estaca et al.<sup>22</sup> Cheng et al, described thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate with emphasis on the antimicrobial and anti inflammatory properties of the ferulic acid.<sup>28</sup> From the literature one can surmise that chitosan has the ability to react with polyphenols to make products with unique applications. These references are also indicating the distinctive properties of products when gelatin is incorporated to make a biopolymer.

This present study investigates the preparation of biopolymers using gelatin, chitosan and the vegetable tannin tara. The solubility of chitosan, optimal conditions necessary for polyphenols to react with gelatin and chitosan, and the physical properties of the resultant products will be reported. The gels are examined for thermal stability and for fluorescence.

## EXPERIMENTAL

### Materials

Commercial Type B gelatin from bovine skin, characterized in this laboratory as 175g Bloom, gallic acid and chitosan (low molecular weight and 75-85% acetylated) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Tara was obtained from Hermann Oak Leather Company (St. Louis, MO). All other chemicals were analytical grade and used as received.

### Methods

#### *Preparation of Gallic Acid- and Tara-modified Gelatin and Chitosan Biopolymer Products*

Chitosan (0-1.0g) was dissolved in 20 mL of 0.15M acetic acid. Gelatin (175g Bloom) (5g) was suspended in 20 ml of water, held for 2 h at room temperature (RT) (25-28°C) and then heated at 45°C for 10 min. When gelatin went into solution, the chitosan solution was added and mixed well. The solution was allowed to sit at RT for 2 h and then stored o/n at 4°C. The solution was then heated at 65°C for 15 min, about 10 ml of water was added and the pH slowly adjusted from 5-10 with NaOH; precipitation of chitosan was continually monitored. Gallic acid (calculated to be 0-5%) and tara (calculated to be 0-6%), both based on weight of total protein and chitosan, were prepared in 10 mL of water. The tara solutions needed to be heated (40°C) to dissolve the product, centrifuged, and the supernatant added with stirring to the protein solutions. After addition of polyphenol to the solution, the mixture was stirred well and sonicated (bath sonicator) for 5 min. Samples to which no tara or gallic acid were added, were run as controls to monitor changes in physical properties. Aliquots (10 mL) of all the reaction mixtures were added to test tubes for melting point determination and 30-mL aliquots were 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°C in a constant temperature bath. 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  $\mu$ L of 1% solution) was added to the remaining treatment solutions as a preservative; the samples were stored at 4°C.

### Analyses

#### *Physical Properties and Molecular Weight Distribution*

Gel strength, melting point, and viscosity of the gel/chitosan/tara-treated solutions were determined as described in a previous publication.<sup>29</sup> Protein molecular weights were estimated as described previously.<sup>30</sup> In summary, SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) was run using precast 4-15 percent gradient gels. A broad range (BRS) calibration standard (Bio-Rad, Hercules, CA),

which contains a mixture of nine proteins ranging in size from 6,500 to 200,000 Da, 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%  $\beta$ -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 -modified gelatin/chitosan biopolymers and unmodified control samples was determined on a Multi-Cell Differential Scanning Calorimeter (DSC) (model CSC-4100) from Calorimetry Sciences Corporation, Lindon, UT, as previously described.<sup>31</sup> In preparation for DSC experiments, unmodified and modified-gelatin/chitosan (100-150 mg) samples were weighed into ampoules and a small amount of distilled water (500  $\mu$ L) was added; the ampoules were sealed and placed in the calorimeter. The calorimeter was programmed to record heat flow as  $\mu$ cal/°C while the temperature was increased from 10°C to 180°C at 1.0°C/min with an equilibration period of 10 min at the start. The temperature at the peak of the calorimetry trace,  $T_p$ , was considered to be an apparent melting temperature.

### **Optical Microscopy (With Epi-fluorescent Attachment)**

Samples of tara, unmodified gelatin/chitosan and gelatin/chitosan modified with 0%, 3%, 4%, and 6% tara, were prepared and then checked for fluorescence using an epi-fluorescent microscope. They were examined using an Eclipse E600 Polarizing Microscope (Nikon Instruments Company, Melville, NY), at 4X magnification, operating in optical mode. The instrument was equipped with a X-Cite™ 120 Fluorescence Illuminator System which was fitted with a metal halide lamp (EXFO Photonic Solutions, Inc., Mississauga, ON, Canada), with two filter cubes or optical blocks, containing epi-fluorescence interference and absorption filter combinations including an excitation filter, dichromatic beamsplitter (often referred to as a mirror), and a barrier (or emission) filter (515-555 nm or 600-660 nm), and with a digital camera (DS-Fi1).<sup>32</sup>

## **RESULTS AND DISCUSSION**

### **Solubility of Chitosan in Presence of Gelatin**

The chitosan product used in these experiments, as described by the supplier,<sup>33</sup> is 75-85% deacetylated, has a molecular weight ranging from 50,000-190,000 Da, and a 1% concentration is soluble in 1% acetic acid. In our initial experiments, we effectively dissolved from 0.1 to 1.0% chitosan in 0.15 M acetic acid. Our next objective was to ensure that solutions containing gelatin and chitosan would be compatible, a necessary step if we wish to ultimately make a biopolymer. We prepared gelatin solutions, they were allowed to swell at RT, and, with heat in a

45°C bath, were solubilized. The gelatin solutions were added to the 0.1 to 1% chitosan solutions, were well mixed, allowed to sit for 2 h, and then heated in a 65°C bath for 15 min; the gelatin and chitosan remained soluble.

### **Effect of pH on Chitosan/gelatin Solutions**

We subsequently determined the concentrations of chitosan that could remain soluble in presence of gelatin at various pH's. Previous research<sup>34</sup> has shown that 0.32% chitosan remains soluble at pH of about 6.0 in the presence of 5-10% w/v gelatin. We tried 0.10 to 0.50% chitosan in the presence of 5% w/v gelatin starting at an initial pH of about 4 and slowly raising the pH to 7.5. The concentrations of chitosan above 0.35% concentration became cloudy. When we raised the pH of a chitosan solution in the absence of gelatin, some cloudiness was seen even at pH 6.0 and the viscosity values above this pH are not reliable for precipitation has begun.

We found previously that the optimal pH for gelatin reactions with gallic acid and tara was 9-10<sup>5,7</sup> as shown in Figure 1.

Gallic acid (2%) was used to modify gelatin, and the physical data (Figure 1), show that as the pH approaches 10, the gel strength decreases and at the same time the melting point and viscosity increase, to the point that neither of the latter two could be determined, for the product did not melt and the viscosity could not be read.

Molecular weight distribution studies (Figure 2) substantiate this for as the pH approaches 10, the bands representing gelatin disappear and at the same time, a band that does not enter the gel appears after pH 6.0, grows more intense, and then basically starts to disappear as the pH approaches 10.0.

### **Effect of Gallic Acid and Tara on Physical Properties of Gelatin/chitosan Solutions**

Chitosan, 0.35% and 5% gelatin were reacted with 2% and 4% gallic acid at pH's from 5-10, 45°C, for 4h.

The physical properties for both concentrations of gallic acid (Figure 3) were for the most part similar, however, at the 4% gallic acid concentration, pH 10, the melting point was higher than 60°C and the gel was irreversible and so viscosity could not be read. The chitosan did not precipitate at these higher pH's. As a control 5% gelatin and 0.35% chitosan concentrations were run in which pH was raised from 5-10. The physical properties of an unmodified gelatin/chitosan solutions (Figure 4) and the curves are similar to gelatin solutions at the same pH's reported by Bello et al.<sup>35</sup>; all parameters could be read, for again, the chitosan had not precipitated.

In the next set of experiments, 4% tara was used to modify 0.35% chitosan/5% gelatin solutions, at pH from 5-10. The results can be seen in Figure 5.

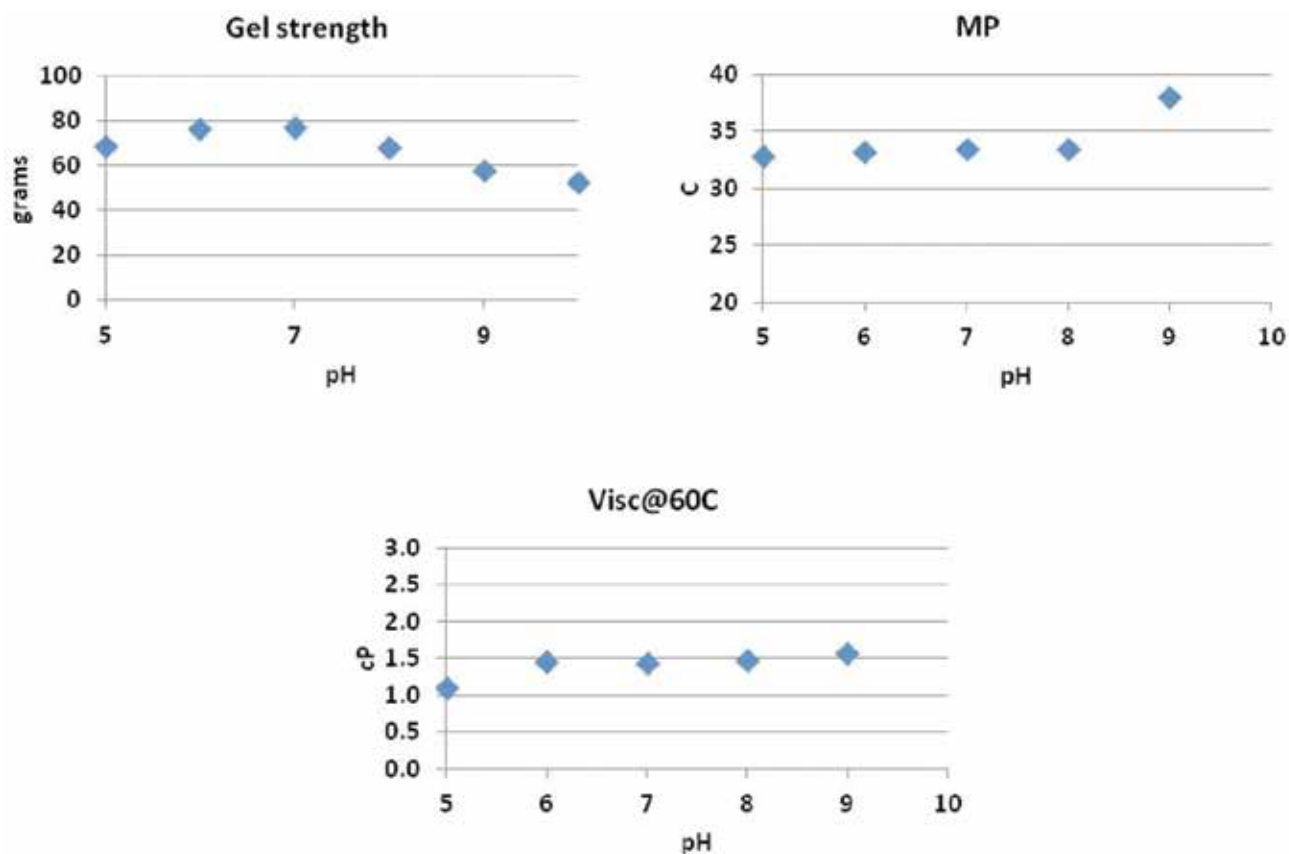


Figure 1. Gel strength, melting point, and viscosity (at 60°C) of 175 Bloom gelatin (5% w/v) treated with 2% gallic acid at pH 5.0-10.0, 45°C for 4 h.

The highest viscosity, the desirable property for filler, was attained at pH of 9.0. In the next set of experiments, the pH (9.0), chitosan (0.35%) and gelatin (5%) remained constant and the concentration of tara was varied from 0-6%. Results showed that the viscosity was optimal at 3-4% concentration of tara (Figure 6).

The samples from the varying tara concentration series were examined by SDS-PAGE for molecular weight distribution and, as seen in Figure 7, the band at the top of the gel, a high molecular weight moiety has begun to disappear around 4% offer of tara, showing that the reaction is becoming more effective. The optimal concentrations of tara (3% and 4%) were run in triplicate to test the reproducibility of the reaction and the results can be seen in Table I. As indicated by the standard deviation, the reaction is quite reproducible.

#### DSC Analyses and Epi-fluorescent Imaging

DSC analyses were run on the 3% and 4% tara-modified gelatin/chitosan samples (Figure 8), as well as on gelatin, chitosan and tara alone. Due to the heterogeneous nature of all samples, the curves are showing many small peaks. As a control, the 0% tara-modified gelatin/chitosan is also shown and the gelatin (32°C) and chitosan (23°C) peaks can be seen. However, when one compares the gelatin and chitosan peaks in the treated 3% and 4% tara samples to the unmodified

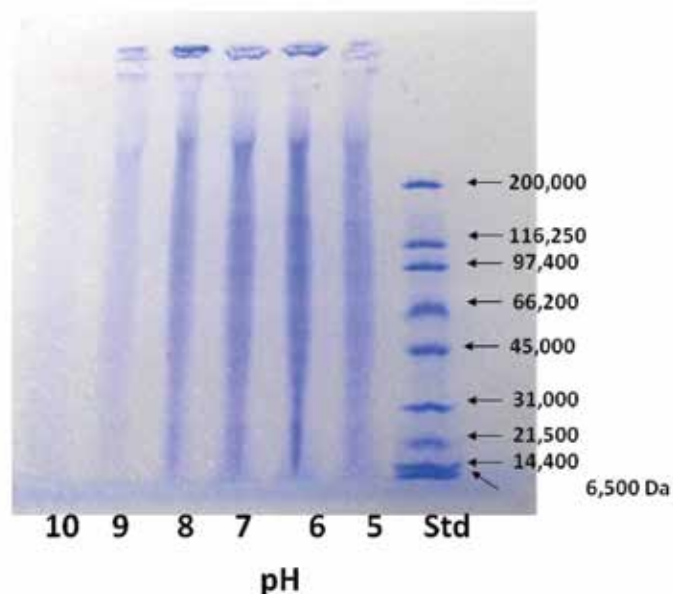


Figure 2. SDS-PAGE of 175 Bloom gelatin (5% w/v) treated with 2% gallic acid at pH 5.0-10.0, 45°C for 4 h; molecular weights are shown in Da.

gelatin and chitosan sample (0% tara), these peaks have disappeared, indicating that a reaction has taken place. Furthermore, in both the 3% and 4% modified samples a new peak is appearing at around 28°C; the peaks contributed by tara (38°C and 45°C) are seen in these samples.

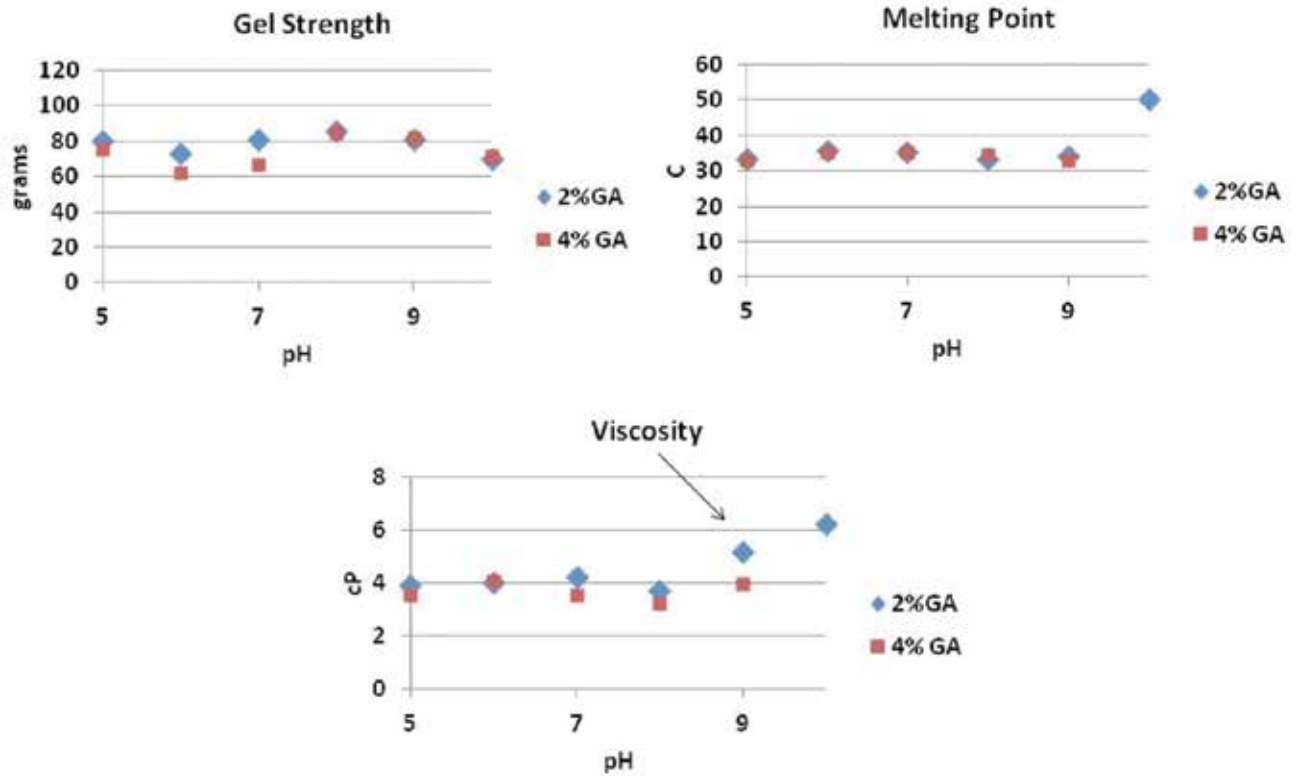


Figure 3. Gel strength, melting point, and viscosity (at 60°C) of chitosan (0.35% w/v) and 175 Bloom gelatin (5% w/v) treated with 2% or 4% gallic acid at pH 5.0-10.0, 45°C for 4 h.

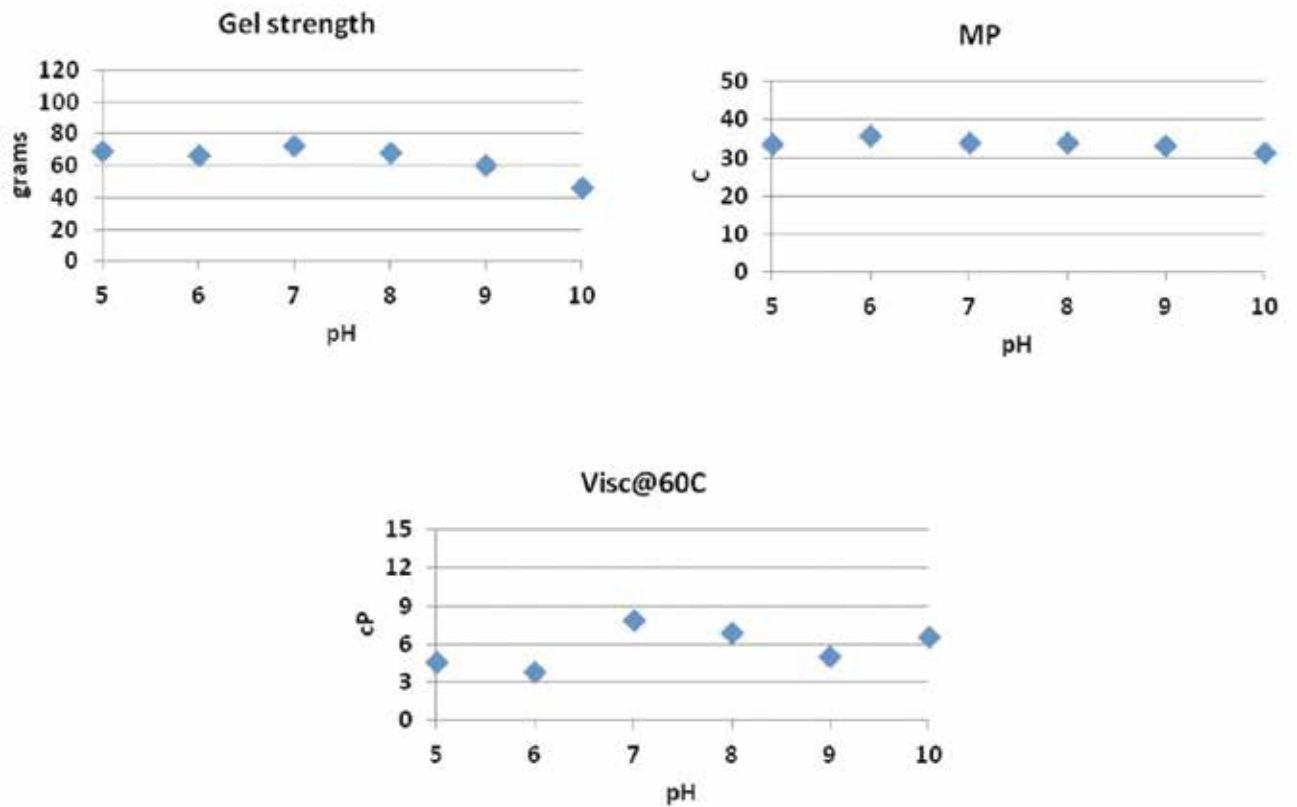


Figure 4. Gel strength, melting point, and viscosity (at 60°C) of chitosan (0.35% w/v) and 175 Bloom gelatin (5% w/v) treated with 0% polyphenol at pH 5.0-10.0, 45°C for 4 h.

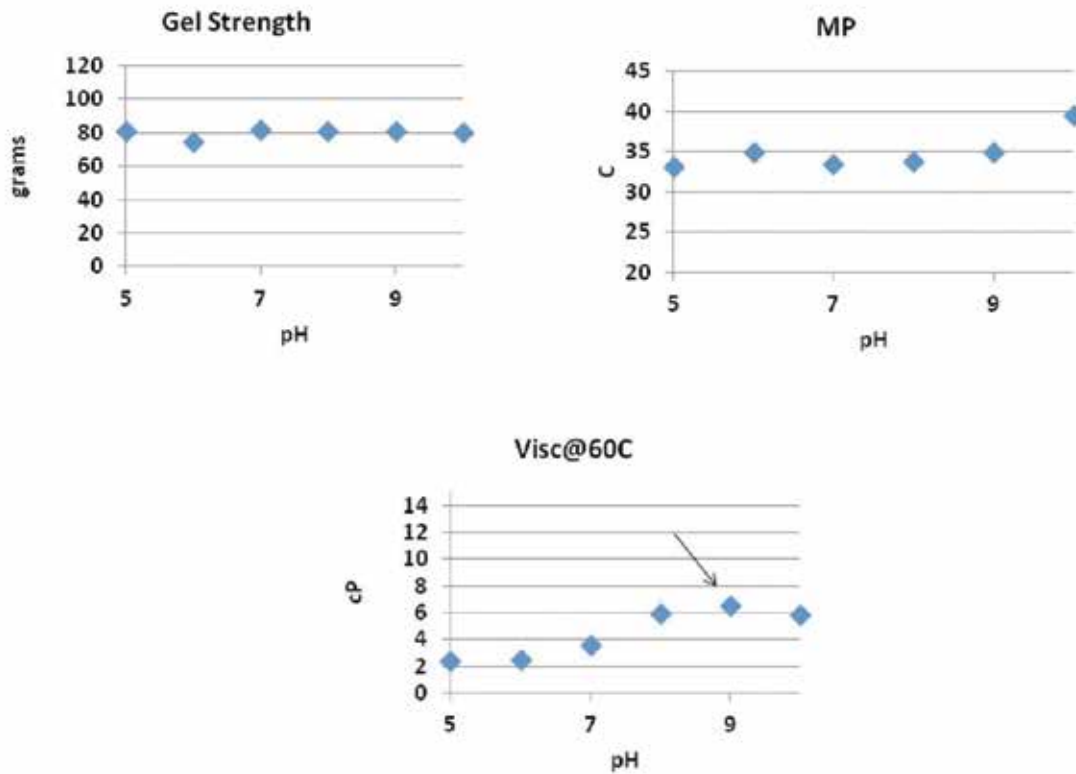


Figure 5. Gel strength, melting point, and viscosity (at 60°C) of chitosan (0.35% w/v) and 175 Bloom gelatin (5% w/v) treated with 4% tara at pH 5.0-10.0, 45°C for 4 h.

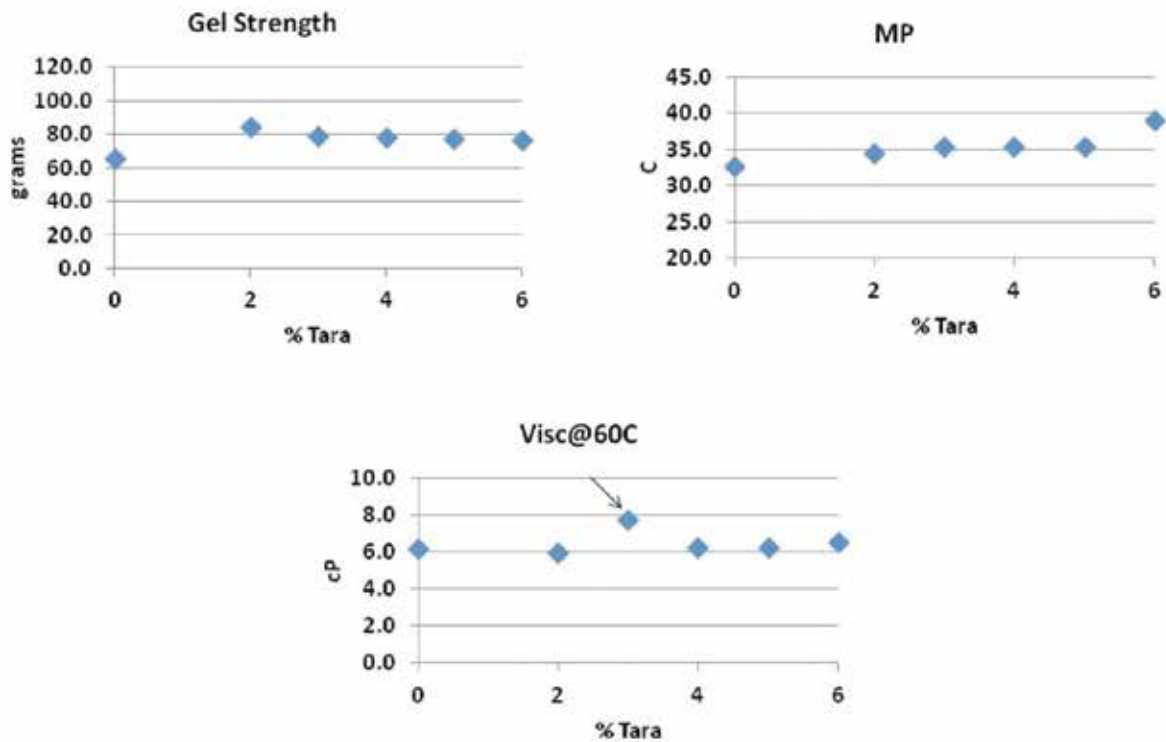


Figure 6. Gel strength, melting point, and viscosity (at 60°C) of chitosan (0.35% w/v) and 175 Bloom gelatin (5% w/v) treated with 0-6% tara at pH 9.0-10.0, 45°C for 4 h.

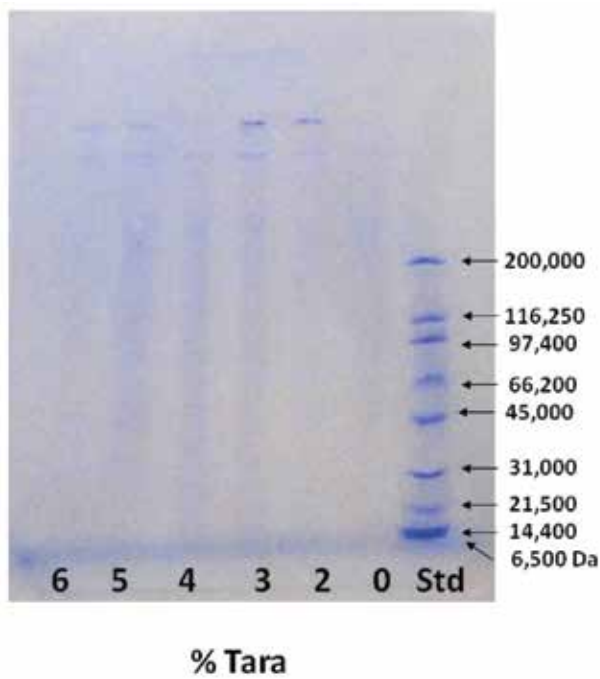


Figure 7. SDS-PAGE of chitosan (0.35% w/v) and 175 Bloom gelatin (5% w/v) treated with 0-6% tara, pH 9.0-10.0, 45°C for 4 h; molecular weights are shown in Da.

**TABLE I**  
**Reproducibility of reaction.\***

	Gel Strength (grams)	Melting Point (°C)	Viscosirt @ 60°C (cP)
3% Tara			
	71.2	35.5	8.27
	77.0	35.5	7.81
	81.8	35.8	5.91
Average	76.7	35.6	7.33
STD	3.8	0.1	0.9
4% Tara			
	77.8	35.8	7.97
	77.3	36.2	6.31
	77.0	36.2	7.28
Average	77.4	36.1	7.19
STD	0.3	0.2	0.6

\*5% gel, 0.35% chit, pH 9-10, 45°C

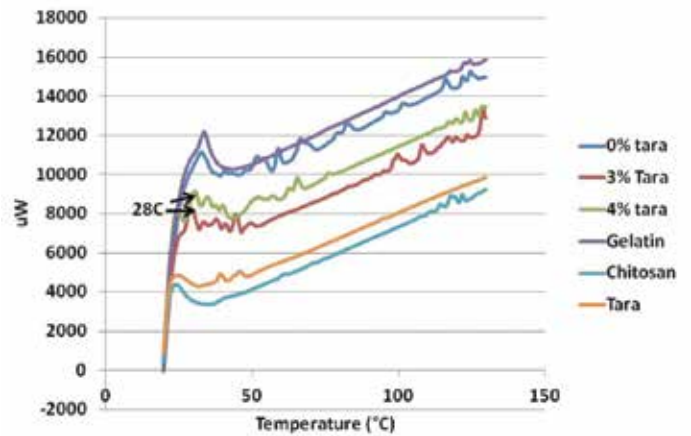


Figure 8. DSC analysis of chitosan (0.35% w/v) and 175 Bloom gelatin (6% w/v) treated with 0, 3, and 4% tara, pH 9.0-10.0, 45°C for 4 h; analyses of tara, gelatin, and chitosan alone are shown.

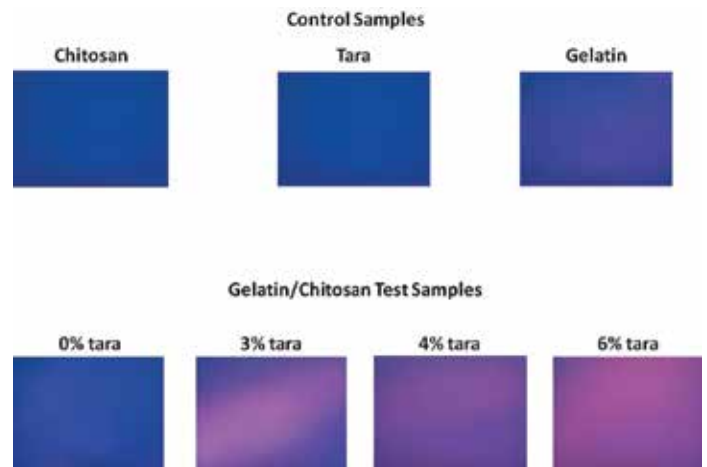


Figure 9. Epi-fluorescent micrographs of chitosan, tara and gelatin, alone and 5% gelatin/0.35% chitosan gel treated with 0%, 3%, 4%, and 6% tara, pH 9.0-10.0, 45°C for 4 h; emission (barrier) filter between 600-660 nm was used.

Epi-fluorescent studies were carried out on products from the tara-gelatin/chitosan study. As controls, chitosan, gelatin and tara alone were examined at 515-550 nm and 600-660 nm emissions. The chitosan and tara control samples which were examined at the 515-550 nm range exhibited no fluorescence, however for gelatin, the control sample has intense fluorescence in this range; the gelatin test samples also fluoresced brightly and no difference could be distinguished between the control and test samples. In the 600-660 nm range (Figure 9), only the gelatin control samples exhibited a very pale pink fluorescence. The gelatin/chitosan samples that were treated with 0%, 3%, 4%, and 6% tara were examined and as the tara concentration increases (Figure 9), the strength of pinkish-purple color gradually intensifies till the 6% offer where the color is almost a pinkish-red. These results are showing that the individual components alone have little or no fluorescence (at these emissions) but when combined and the

concentration of tara is increased, the corresponding increase in fluorescence, particularly in the 600-660nm range, is significant. What part the modified chitosan is contributing to this fluorescence is difficult to determine, for unlike gelatin which we can modify with tara at pH 9-10 and look at the resulting fluorescence, chitosan cannot be treated at this pH (without being in the presence of gelatin) for it will precipitate.

However, if one examines these results from the fluorescence study, along with the physical properties, molecular weight distribution, and the DSC data, they are all indicating that a reaction is taking place with the gelatin, chitosan and tara and this reaction is being driven by the concentration of the tara.

## CONCLUSIONS

Gelatin is in short supply and is increasingly more expensive. There is an urgent need to find a substitute that could be combined with the gelatin, and by partially replacing and reducing the amount of gelatin required, the potential exists that the new products would retain the desired characteristics of gelatin products. We have shown that it is possible to make a product from gelatin and chitosan that would give properties similar to gelatin alone (e.g. viscosity). There are advantages (plentiful supply) and disadvantages (insolubility) for use of chitosan in biopolymers. One has to balance, at appropriate pH, the reaction of gelatin/chitosan with polyphenol, and with solubility of chitosan. Most importantly it was shown that chitosan, which normally precipitates in solutions over pH 6, will remain in solution at higher pH's in the presence of gelatin. Preliminary results therefore have shown that we have made a product with good viscosity and interesting functional properties that may have application in the preparation of films, coatings, flocculants or fillers.

## REFERENCES

1. <http://www.ft.com/intl/cms/s/0/1f01d088-4900-11e1-954a-00144feabdc0.html#axzz1xsCCKbIO> (accessed 05/20/15)
2. Zhang, Z.-Q., Pan, C.-H., Chung, D.; Tannic acid cross-linked gelatin–gum arabic coacervate microspheres for sustained release of allyl isothiocyanate: Characterization and in vitro release study. *Food Research International* **44**, 1000-1007, 2011.
3. Strauss, G., and Gibson, S.M.; Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids* **18**, 81-89, 2004.
4. Mathew, S., and Abraham, T.E.; Characterisation of ferulic acid incorporated starch–chitosan blend films. *Food Hydrocolloids* **22**: 826-835, 2008.

5. Taylor, M.M., Lee, J., Bumanlag, L.P., Latona, R., Brown, E.M., and Liu, C.-K.; Preparation and characterization of polyphenol-modified gelatin products. *JALCA* **107**, 51-59, 2012.
6. Taylor, M.M., Medina, M., Lee, J., Bumanlag, L.P., Brown, E.M., and Liu, C.-K.; Treatment of wet blue with quebracho-modified gelatin biopolymer products. *JALCA* **107**, 416-421, 2012.
7. Taylor, M.M., Lee, J., Bumanlag, L.P., Latona, R.J., Brown, E.M., and Liu, C.-K.; Preparation and evaluation of tara-modified proteins. *JALCA* **108**, 16-22, 2013.
8. Taylor, M.M., Medina, M., Lee, J., Bumanlag, L.P., Latona, N.L., Brown, E.M., and Liu, C.-K.; Treatment of hides with tara-modified protein products. *JALCA* **108**, 438-444, 2013.
9. Taylor, M.M., Lee, J., Bumanlag, L.P., Latona, R.J., Brown, E.M., and Liu, C.-K.; Biopolymers produced from gelatin and whey protein concentrate using polyphenols. *JALCA* **109**, 82-88, 2014.
10. Taylor, M.M., Bumanlag, L.P., Lee, J., Latona, N.L., Brown, E.M., and Liu, C.-K.; Utilization of agricultural by-products to partially replace gelatin in preparation of products for leather. *JALCA* **110**, 13-18, 2015.
11. Bhatnagar, A., Sillanpää, M.; Applications of chitin- and chitosan-derivatives for the detoxification of water and wastewater — A short review. *Advances in Colloid and Interface Science* **152**, 26-38, 2008.
12. Zeng, D. W., J., Kennedy, J.F.; Application of a chitosan flocculant to water treatment. *Carbohydrate Polymers* **71**, 135-139, 2008.
13. No, H. K., Meyers, S.P., Prinyawiwatkul, W., and Xu, Z.; Applications of chitosan for improvement of quality and shelf life of foods: A Review. *Journal of Food Science* **72**, R87-R100, 2007.
14. Gómez-Estaca, J., López de Lacey, A., López-Caballero, M.E., Gómez-Guillén, M.C., Montero, P.; Biodegradable gelatine chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. *Food Microbiology* **27**, 889-896, 2010.
15. Gómez-Estaca, J., Lopez de Lacey, A., Gómez-Guillén, M.C., López-Caballero, M.E., and Montero, P.; Antimicrobial activity of composite edible films based on fish gelatin and chitosan incorporated with clove essential oil. *Journal of Aquatic Food Product Technology* **18**, 46-52, 2009.
16. Sun, X., Wang, Z., Kadouh, H., Zhou, K.; The antimicrobial, mechanical, physical and structural properties of chitosan–gallic acid films. *LWT - Food Science and Technology* **57**, 83-89, 2014.
17. Siripatrawan, U., and Harte, B.R.; Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids* **24**, 770-775, 2010.

18. Yen, M.-T., Yang, J.-H., Mau, J.-L.; Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers* **74**, 840-844, 2008.
  19. Dash, M., Chiellini, F., Ottenbrite, R.M., Chiellini, E.; Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science* **36**, 981-1014 2011.
  20. Qin, Y.-Y., Yang, J.-Y., Lu, H.-B., Wang, S.-S., Yang, J., Yang, X.-C., Chai, M., Li, L., Cao, J.-X.; Effect of chitosan film incorporated with tea polyphenol on quality and shelf life of pork meat patties. *International Journal of Biological Macromolecules* **61**, 312-316, 2013.
  21. Wang, L., Dong, Y., Men, H., Tong, J., Zhou, J.; Preparation and characterization of active films based on chitosan incorporated tea polyphenols. *Food Hydrocolloids* **32**, 35-41, 2013.
  22. Gómez-Estaca, J., Gómez-Guillén, M.C., Fernández-Martín, F., Montero, P.; Effects of gelatin origin, bovine-hide and tuna-skin, on the properties of compound gelatin–chitosan films. *Food Hydrocolloids* **25**, 1461-1469, 2011.
  23. Rivero, S., García, M.A., and Pinotti, A.; Crosslinking capacity of tannic acid in plasticized chitosan films. *Carbohydrate Polymers* **82**, 270-276, 2010.
  24. Rivero, S., García, M.A., and Pinotti, A.; Composite and bi-layer films based on gelatin and chitosan. *Journal of Food Engineering* **90**, 531-539, 2009.
  25. Gómez-Guillén, M. C., Pérez-Mateos, M., Gómez-Estaca, J., López-Caballero, E., Giménez, P., and Montero, B.; Fish gelatin: a renewable material for developing active biodegradable films. *Trends in Food Science & Technology* **20**, 3-16, 2009.
  26. Wang, S., Marcone, M.F., Barbut, S., Lim, L.-T.; Fortification of dietary biopolymers-based packaging material with bioactive plant extracts. *Food Research International* **49**, 80-91, 2012.
  27. Rocasalbas, G., Francesko, A., Touriño, S., Fernández-Francos, X., Guebitz, G.M., Tzanov, T.; Laccase-assisted formation of bioactive chitosan/gelatin hydrogel stabilized with plant polyphenols. *Carbohydrate Polymers* **92**, 989-996, 2013.
  28. Cheng, Y.-H., Yang, S.-H., Liu, C.-C., Gefen, A., Lin, F.-H.; Thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate. *Carbohydrate Polymers* **92**, 1512-1519, 2013.
  29. Taylor, M.M., Cabeza, L.F., Marmer, W.N., and Brown, E.M.; Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. I. Physical Properties. *JALCA* **96**, 319-332, 2001.
  30. Taylor, M.M., Marmer, W N., and Brown, E M.; Molecular weight distribution and functional properties of enzymatically modified commercial and experimental gelatins. *JALCA* **99**, 129-140, 2004.
  31. Ding, K., Taylor, M. M., and Brown, E. M.; Effect of genipin on the thermal stability of hide powder. *JALCA* **101**, 362-367, 2006.
  32. <http://www.microscopyu.com/articles/fluorescence/filtercubes/filterindex.html> (accessed 05/20/15)
  33. <http://www.sigmaaldrich.com/catalog/product/aldrich/448869?lang=en&region=US> (accessed 05/20/15)
  34. Chen, T., Embree, H.D., Brown, E.M., Taylor, M.M., Payne, G.F.; Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. *Biomaterials* **24**(17): 2831-2841, 2003.
  35. Bello, J., Bello, H.R., and Vinograd, J.R.; The mechanism of gelation of gelatin. The influence of pH, concentration, time and dilute electrolyte on the gelation of gelatin and modified gelatins. *Biochimica et Biophysica Acta* **57**, 214-221, 1962.
-