# Evaluation of Polymers Prepared from Gelatin and Casein or Whey as Potential Fillers[†,§]

*by*

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## Abstract

We recently demonstrated that fillers could be formed inside leather when gelatins alone or mixed proteins, such as gelatin and casein or gelatin and whey, were added to wet blue that had been pretreated with microbial transglutaminase. To monitor these reactions, we had added fluorescently labeled proteins to stock solutions and examined the resultant filled leather using a microscope equipped with an epi-fluorescent attachment. In this present study, based on our understanding of the effect that enzyme modification will have on physical properties of crosslinked proteins, we polymerized potential filler products prior to their addition to the wet blue. We characterized the products with respect to their physical properties and molecular weight distribution (degree of polymerization). These products were applied to wet blue and evaluated, again using fluorescent labels to monitor their filling capability. It was shown that the proteins were evenly distributed throughout the hide and, more importantly, were not removed during the washing steps. Micrographs showing the location of fillers using fluorescent labels are presented.

## Abstracto

Demostramos recientemente que los rellenantes podrían formarse dentro del cuero cuando las gelatinas solas o las proteínas mezcladas, tales como gelatina y caseína o gelatina y suero lacteo, fueron agregadas al wet blue que había sido pre-tratado con transglutaminasa microbiana. Para seguir estas reacciones, hemos agregado las proteínas con etiquetas fluorescentes a las soluciones comunes y hemos examinado el cuero resultante rellenado usando un microscopio equipado de un accesorio epi-fluorescente. En el presente estudio, basado en nuestra comprensión del efecto que la modificación de la enzima tendrá sobre las características físicas de proteínas reticuladas, prepolimerizamos productos rellenantes potenciales antes de su adición al wet blue. Caracterizamos los productos con respecto a sus propiedades físicas y la distribución del peso molecular (grado de polimerización). Estos productos fueron aplicados a wet blue y evaluados, otra vez usando etiquetas fluorescentes para observar su capacidad de relleno. Fue demostrado que las proteínas fueron distribuidas uniformemente a través de la piel y, más importante, no fueron removidas durante los procesos de lavado. Los micrográficos que demuestran la localización de los rellenantes usando etiquetas fluorescentes se presentan.

## Introduction

In an earlier publication from this laboratory, the use of glutaraldehyde-modified gelatin as a filling agent was described[1]. In these experiments, a fluorescent label was attached to the gelatin before reaction with the crosslinking agent and the resulting product was then added to blue stock; penetration was monitored using a microscope equipped with an epi-fluorescent attachment. The results demonstrated that the polymerized gelatin had indeed entered the loose areas of the hide, acted as a filler, and more importantly, did not wash out after further processing. However, a drawback to the glutaraldehyde modification is that the glutaraldehyde is potentially toxic.

In a more recent study[2], we expanded on the use of gelatins as fillers by examining whether the extensively reported[3-9] enzymatic polymerization of gelatin using environmentally benign and relatively inexpensive microbial transglutaminase would effectively produce filled products similar to those described in chemical modification. In our study, the gelatin was tagged with a fluorescent label, added to blue stock that

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had been pretreated with microbial transglutaminase and the reaction, inside the leather, created a product that would act as a filler. The efficiency of the reaction was again monitored by epi-fluorescent microscopy.

Biopolymers, formed by the enzymatic crosslinking of dissimilar proteins, have the potential for generating novel products. Many reports have appeared on the properties of a variety of biopolymers synthesized by enzymatic treatment[10-18]. Based on this reported research, we designed experiments in which we enzymatically reacted gelatin with casein[19] or whey proteins[20] ; after characterization, we found that unique, highly polymerized products were obtained. We then examined whether these enzymatically treated mixed proteins could also be effective fillers. Casein and whey protein isolate (WPI) were labeled as well as gelatin and, after enzyme assisted reaction inside the blue stock, the resulting filled products were examined using epi-fluorescent microscopy[2,20]. It was shown that indeed these mixed protein products could also be used effectively as fillers, were bound to the leather, and would not easily be removed during further processing.

In this present study, we investigated whether protein combinations could first be enzymatically polymerized (similar to the glutaraldehyde studies), then added to blue stock to give the same filling effect as was found in previous studies. Advantages to producing the products outside the leather would be to allow us to characterize the products with respect to solubility, thermal stability, viscosity and molecular weight distribution (degree of polymerization). To further insure the polymer's attachment, the blue stock was pretreated with enzyme. To evaluate the filling properties of the different biopolymer products, fluorescent labels were attached to the proteins prior to polymerization. After treatment of the blue stock, the filled leather was then examined using the epi-fluorescent microscope. Our ultimate goal is to produce products from these reactions between dissimilar proteins (from renewable resources) that have the potential to be used in leather processing as economical replacements to conventional high cost protein products, such as casein, and petroleum-based fillers.

## EXPERIMENTAL

### Materials

Activa TG-TI, a microbial transglutaminase (mTgase) (approximately 100 units/g) containing maltodextrin as a carrier, with activity from pH 4.0 to 9.0, at 0 to 70°C, was obtained from Ajinomoto USA, Inc. (Paramus, NJ), stored at 4°C in a sealed package, and used without further preparation. Commercial Type B gelatin from bovine skin, characterized in this laboratory as 175 grams Bloom, was obtained from Fisher (Fairlawn, NJ). Dithiothreitol (DTT) and whole whey, 11% protein and approximately 65% lactose, were obtained from Sigma (St. Louis, MO). Sodium caseinate (Alanate® 180) and Alacen™ 895 (whey protein isolate or WPI), containing 93.2% protein, were generously supplied by NZMP (formerly New Zealand Milk Products) (Lemoyne, PA). Alexa Fluor® 488 and Alexa Fluor® 568 protein labeling kits were obtained from Molecular Probes, Inc. (Eugene, OR). Chrome tanned blue stock was purchased from a local tannery. All other chemicals were analytical grade and used as received.

### Preparation of fluorescently labeled conjugates

The biopolymers were composed of either gelatin/casein or gelatin/whey or WPI; in order to observe both proteins' contribution to the biopolymer (and migration through the leather), labels with different emissions were added to gelatin and to casein or WPI. An Alexa Fluor® 488 protein labeling kit (procedure described in Molecular Probes, Product Information, November 13, 2003) was used to attach a fluorescent label (absorbance at 494 nm and fluorescence emission at 519 nm) to 175 Bloom gelatin. An Alexa Fluor® 568 labeling kit was used to attach a fluorescent label (excitation at 577 nm and fluorescence emission maxima at 603 nm) to casein and WPI according to the procedure described in Molecular Probes, Product Information, August 23, 2004. Each protein (2 mg/ml) was prepared in phosphate buffered saline (PBS), pH 7.2; gelatin was swollen for two hours and then heated at 65°C until melted. To 0.5 ml of the protein solution was added 50 µl of 1 M sodium bicarbonate and the protein solution was added to reactive dye and stirred for 1 h at room temperature (RT). The reaction mixture was added to a column containing Bio-Rad BioGel P-30 suspended in PBS. The sample was eluted using the PBS and the location of the conjugate on the resin was monitored using a handheld UV lamp. The first fluorescent band to elute was collected and the second band (unreacted label) was discarded. The labeled protein was protected from light, stored at 4°C, and aliquots were added to stock protein solutions immediately before their use in the preparation of biopolymers using mTgase.

### Preparation of fluorescently labeled polymer products

Gelatin samples (175 Bloom; 6 g) alone or in combination with sodium caseinate, WPI or whey (1.2 g) were suspended in water (44 ml), and allowed to swell for about 2 h at RT; they were stored overnight at 4°C. They were placed in a bath at 65°C until dissolved; at this point Alexa Fluor® 488 labeled gelatin and Alexa Fluor 568 labeled casein or WPI were added to the solutions (labeled WPI was added to whole whey solutions). Control samples and unlabeled modified biopolymer samples (enzyme additions similar to labeled protein studies), were run to monitor changes in physical properties and to insure that the biopolymers had suitable melting points and viscosities to be used as fillers. To samples that contained WPI or whey, 0.5% DTT was added. The pH was adjusted to 6.5-7.0 with 1 N NaOH. Varying concentrations of microbial transglutaminase (calculated to be 3 units/g of total protein for biopolymer reactions or 8 units/g based on weight of gelatin) were prepared in 10 ml of water and these solutions were added with stirring to the protein solutions to give a final protein concentration of 10% w/w for gelatin and 2% w/v for sodium caseinate, WPI and whey. Aliquots (10 ml) of the reaction mixture were added to test tubes for melting point determination and the remainder of each solution was poured into appropriate containers for determining

gel strength. These samples were warmed to 50°C in a shaker bath and the reaction was carried out for 4 h. The enzyme was inactivated by heating the reaction products at 90°C for 10 min. 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 was added as a preservative and the samples were stored, tightly covered, at 4°C until use.

**Application of filler to wet blue leather**

Wet blue samples (4 pieces/drum, ~25g each, 400% float) were washed by tumbling in a Dose drum (Model PFI 300-34, Dose Maschinenbau GmbH, Lichtenau, Germany) for 30 min at 50°C, drained and refloated in a 1% sodium bicarbonate solution (400% float). The samples were tumbled at ambient temperature (25-28°C) until the pH stabilized. The float was drained, mTgase (5% based on wet weight of hide) was added, and samples were tumbled for 1 h at ambient temperature. The float was drained and labeled polymerized gelatin alone or in combination with labeled casein, WPI, or whey solutions (diluted to give a 400% float based on wet hide weight) was added to the drums. The samples were then tumbled for 1 h at ambient temperature and then for 4 h at 50°C. The pH was then adjusted to 3.5-4.0 with 4.0 M acetic acid. The floats were drained and the samples were washed twice for 10 min at 50°C (400% float), drained, patted dry and stored at 4°C in a dark place until examination under the microscope.

**Analyses**

***Physical properties and molecular weight distribution***

Gel strength, melting point, viscosity, and molecular weight distribution (by SDS-PAGE) of the enzyme-treated proteins were determined as described in previous publications[21, 22].

***Optical microscope equipped with Epi-fluorescent attachment***

The treated blue stock samples were sectioned, using a razor (grain to flesh) and mounted onto a glass slide. They were examined using an Eclipse 6600 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 and including an excitation filter, dichromatic beamsplitter (often referred to as a mirror), and a barrier (or emission) filter[23], and with a digital camera (DXM 1200).

***Scanning electron microscopy (SEM)***

The treated blue stock samples were cut into small strips (6.5 cm x 1 cm) and freeze-dried. Two pieces (1.5 mm) were cut from each of the dry samples and were mounted onto the surfaces of carbon adhesive tabs with the help of Duco cement. After drying for at least 1 h, silver paint was applied to the exposed surface area around the samples. The samples were sputter-coated with a thin layer of gold using a Scancoat Six Sputter coater (180 sec). Samples were viewed using a Quanta 200 FEG Environmental Scanning Electron microscope, FEI Company (Hillsboro, OR) in high vacuum-secondary electron imaging mode at an accelerating voltage of 10 kV (spot size 3.0, pressure 0.3 torr). Digital images were collected at 50, 250 and 1000x magnification.

## RESULTS AND DISCUSSION

**Preparation of labeled conjugates**

Prior to running the polymerization reactions, the proteins to be used in this study were labeled with fluorescent dyes so that the filler products could be monitored using an epifluorescent

**TABLE I**
**Physical Properties of Biopolymers**

| Samples | Gel Strength (g) | MP (°C) | Viscosity (cP) |
|---|---|---|---|
| Gelatin[a] | | | |
| Control | 447.4 | 35.7 | 6.33 |
| Test (8u[b]) | 432.0 | 40.5 | 11.09 |
| Gelatin/casein[a] | | | |
| Control | 235.3 | 33.0 | 7.46 |
| Test (3u[b]) | 402.6 | 39.8 | 15.02 |
| Gelatin/WPI[a] | | | |
| Control | 361.4 | 35.2 | 6.87 |
| Test (3u[b])[c] | 350.2 | 40.1 | 26.69 |
| Gelatin/whey[a] | | | |
| Control[c] | 395.5 | 35.6 | 6.52 |
| Test (3u[b])[c] | 365.0 | 38.4 | 15.32 |

[a]Concentrations: Gelatin = 10% w/w; casein, WPI, and whey = 2% w/v
[b]u=units enzyme/g protein
[c]n =2

microscope. Gelatin, in all reaction combinations, was labeled with Alexa Fluor 488®, which has an emission at 519 nm, and casein and WPI were labeled with Alexa Fluor 568®, which has an emission at 603 nm. WPI was used in the experiment in which whey and gelatin were combined, since the whey contained only 11% protein along with 65% sugar and we were concerned that the labeling may be ineffective on this low protein concentration. These conjugates were prepared, and then were kept at 4°C tightly covered until use. Just prior to polymerization with the enzyme, the labeled proteins were added to the stock solutions. These labeled samples were involved in the analyses described below.

**Physical properties**

Based on the results from data found in previous experiments on the interactions between enzyme-modified gelatin and casein, WPI or whey[19, 20] we identified products that would be liquid at approximately 35-40°C and would possess a low viscosity. Using microbial transglutaminase as the catalyst, we prepared biopolymers from these preferred mixtures of proteins and, before application to blue stock, we characterized the products by examining their physical properties (gel strength, melting point, and viscosity). Table 1 summarizes the results of these analyses. In an earlier paper[2] we described the preparation of a filler from the enzymatic polymerization of gelatin and these data are included for comparison. The data show, that when a 10% w/w concentration of gelatin is treated with 8 units/g of mTgase, there is no significant effect on gel strength, the melting point increases from 35.7°C to 40.5°C and the viscosity increases from 6.33 to 11.09 cP.

We had shown that when gelatin is combined with varying amounts of casein and then modified with mTgase, biopolymers with unique properties, such as improved physical properties and high degree of polymerization, were produced[19]. Based on this information, we prepared a biopolymer in which 10% gelatin and 2% sodium caseinate were mixed and then reacted with 3 units of mTgase. The physical properties of the resultant product, along with a control (unmodified) sample, are shown in Table 1. If one compares the gel strength of the unmodified gelatin alone with that to which 2 % sodium caseinate has been added (with no enzyme), one can see that the mixed protein solution has a lower gel strength than the one with gelatin alone; this phenomenon has been observed in other mixed protein solutions and is probably due to a dilution of the gelatin. The gel strength of the modified sample increased approximately 170% over the control; the melting point and viscosity both increased respectively, the former from 33.0°C to 39.8°C and the latter from 7.5 cP to 15.0 cP.

We have shown that whey or WPI in combination with gelatin can react with mTgase to give unique products with altered physical properties[20]. However, before reaction with the enzyme, whey needs to be pretreated with a reductant[10,24,25] most commonly DTT, in order to break the disulfide bonds and allow the reactive groups to be accessible to the enzyme. To prepare a product that could possibly be used as a filler, we combined 10% gelatin and 2% WPI or 2% whey and the proteins were then reacted with 3 units of mTgase. The physical properties of the resultant products are shown in Table 1. The gel strength of the unmodified gelatin/WPI and gelatin/whey mixtures are lower than gelatin alone, again probably due to dilution of the gelatin; interestingly, the gel strengths of the modified combinations have not shown an increase as was seen in the gelatin/casein combination.

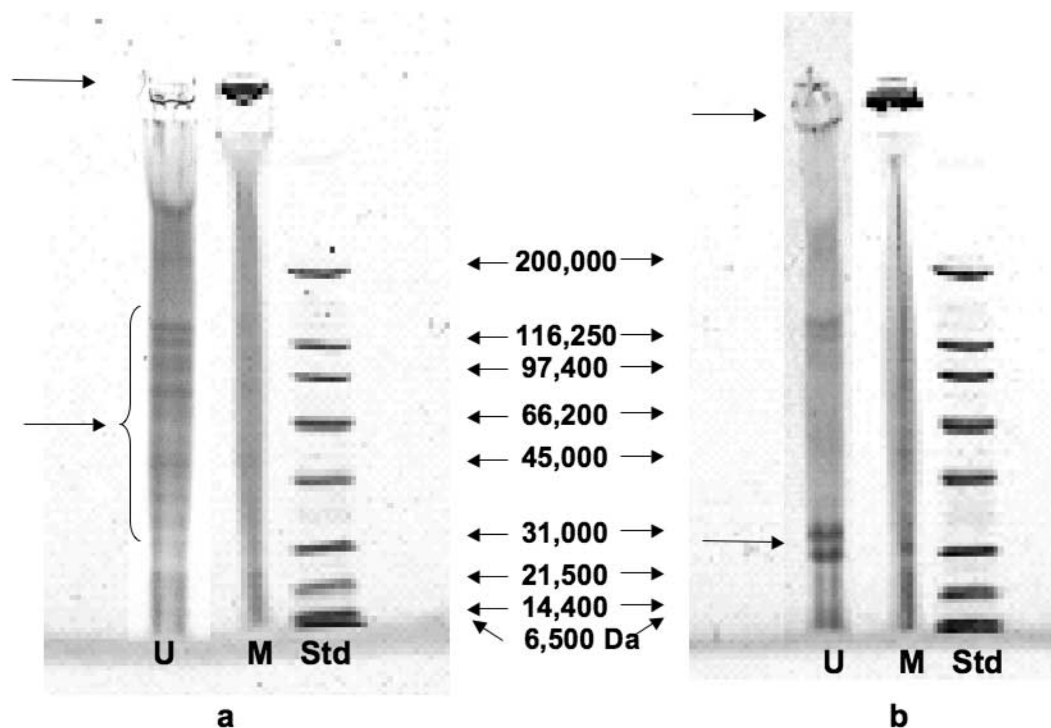



Figure 1. - SDS-PAGE of 10% gelatin with 8 units of mTgase (a) and 10% gelatin with 2% sodium caseinate and 3 units of mTgase (b) ("U" is unmodified or control sample and "M" is modified sample; molecular weights are shown in Da).

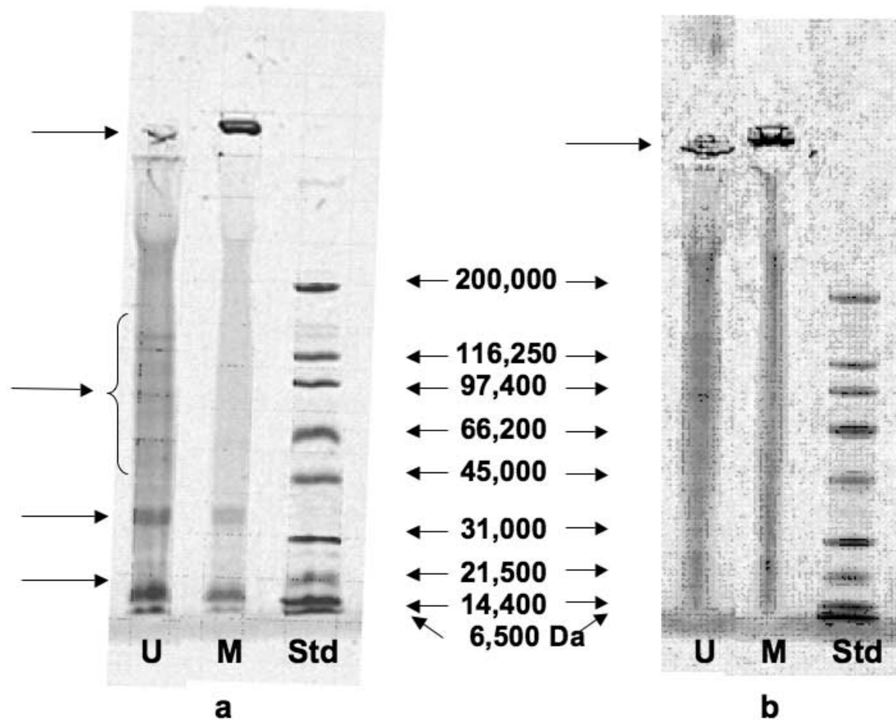



Figure 2. - SDS-PAGE of 10 % gelatin with 2% WPI and 3 units of mTgase (a) and 10% gelatin with 2% whey and 3 units of mTgase (b) ("U" is unmodified or control sample and "M" is modified sample; molecular weights are shown in Da).

However, the melting point of the modified gelatin/WPI has increased from 35.2 to 40.1°C and that of the modified gelatin/whey from 35.6 to 38.4°C. Furthermore, the viscosity of gelatin/WPI has increased from 6.87 to 26.69 cP and that of the modified gelatin/whey from 6.52 to 15.32 cP. We see a more substantial change in the physical properties of the WPI combinations over the whey; the latter has only about 11% protein to react with the gelatin and enzyme while the former is composed of approximately 94% protein.

**Molecular weight distribution**

SDS-PAGE analysis, to determine molecular weight distribution, was run on all samples and the results are shown in Figures 1 and 2. Figure 1a shows the gel of a 10% w/w gelatin solutions modified with 8 units of mTgase. A control (unmodified) sample is also shown for comparison. The control sample shows only gelatin bands in the 31,000 Da to 116,000 Da range; for the modified ample, these bands are less dense and an additional high molecular weight band that has not entered the gel is observed, a phenomenon we have seen previously[22,26].

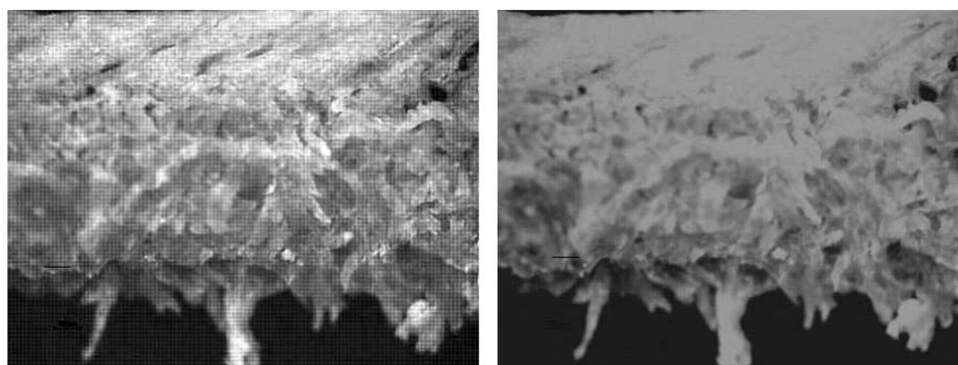
Figure 1b shows the molecular weight distribution of modified and unmodified 10% w/w gelatin and 2% w/w sodium caseinate combination. Unlike gelatin, casein has distinct bands, which can be seen in the 21,500 to 31,000 molecular weight range in the control sample. In the modified samples these bands have become lighter and at the same time, a high molecular weight moiety has appeared that has not entered the gel. Previously[19], we had found that when gelatin and casein were combined and reacted with mTgase, the enzyme appeared to react preferentially with the casein and from the appearance of this gel, it has done this again, with the gelatin bands only slightly diminished.

Figures 2a and 2b are showing the molecular weight distribution of gelatin/WPI and gelatin/whey combinations, both modified and unmodified. Since WPI has much more protein than the whey, one can see in Figure 2a the distinct bands of this protein at approximately 14,400 Da and again at approximately 31,000 Da. The gelatin's contribution can be seen mainly in the 45,000 to 116,000 Da range. In the test sample, the bands contributed by both the WPI and gelatin have diminished and again a high molecular weight band that does not enter the gel has appeared. In Figure 2b, the gelatin/whey combination is shown and it is hard to see contribution by the whey, since there is so little protein in the sample and the gelatin's contribution is again diffused over a wide molecular weight range. The major change is the appearance of the high molecular weight moiety that does not enter the gel, as seen in the modified sample in Figure 2b.

In all combinations, these molecular weight distributions are correlating with the physical properties as described above. They are indicating that change has taken place in the products, in which the molecular weight has increased along with viscosity and melting point, but not to the point that the samples cannot be used as fillers, e.g. samples that had too high viscosity, or product that would not melt.

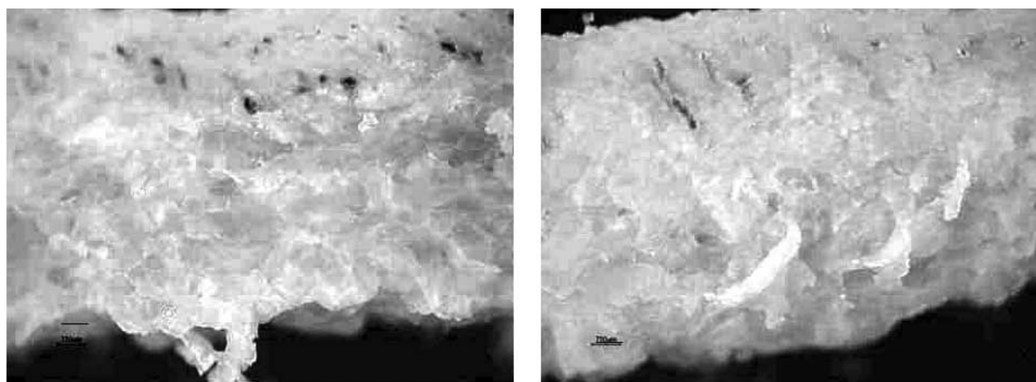
**Application of fillers to wet blue**

The blue stock pieces were washed thoroughly several times. When the appropriate pH for optimal enzyme activity was reached (about 6.5-7.0 after pretreatment with 1% $NaHCO_3$), the floats were drained and mTgase was added; the samples were then tumbled, at ambient temperature, for 1 h. The purpose of this step was to allow the enzyme to be distributed uniformly inside the hide pieces but not react to a great extent with the hide because the temperature was not optimal.



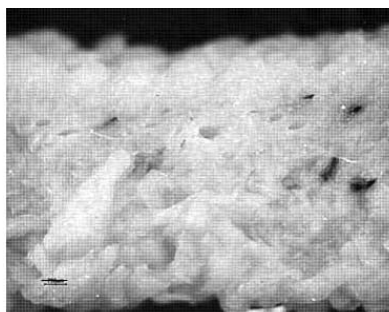
a b

Figure 3. - Epi-fluorescent microscopic images of blue stock treated with pH-adjusting agents, emission at 519 nm (a) and emission at 603 nm (b). ( - = 750 μm).

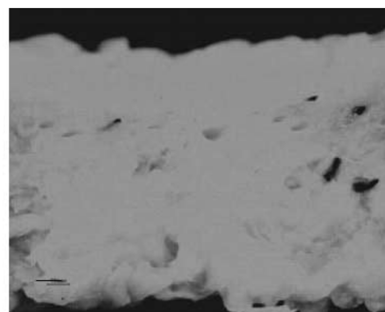


a b

Figure 4. - Epi-fluorescent microscopic images of blue stock treated with a polymer prepared from gelatin (offer 5% gelatin, based on hide weight) and mTgase; gelatin was labeled with Alexa Fluor 488, emission at 519 nm (a and b). ( - = 750 μm).



a



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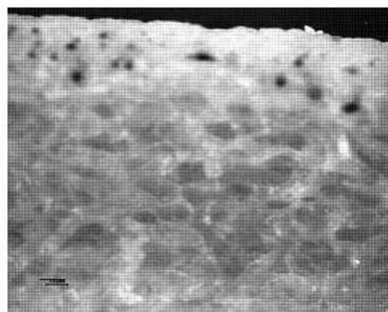

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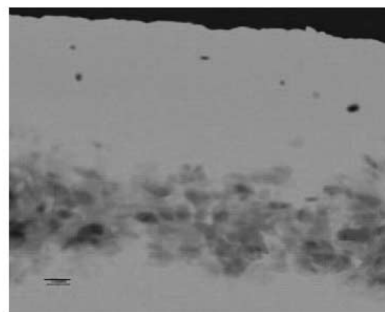

d

Figure 5. - Epi-fluorescent microscopic images of blue stock treated with a polymer prepared from gelatin, sodium caseinate and mTgase (offer 5% gelatin, 1% sodium caseinate based on weight of hide); (a and c) 519-nm emission from labeled gelatin, (b and d) 630-nm emission from labeled casein. ( - = 750 μm).

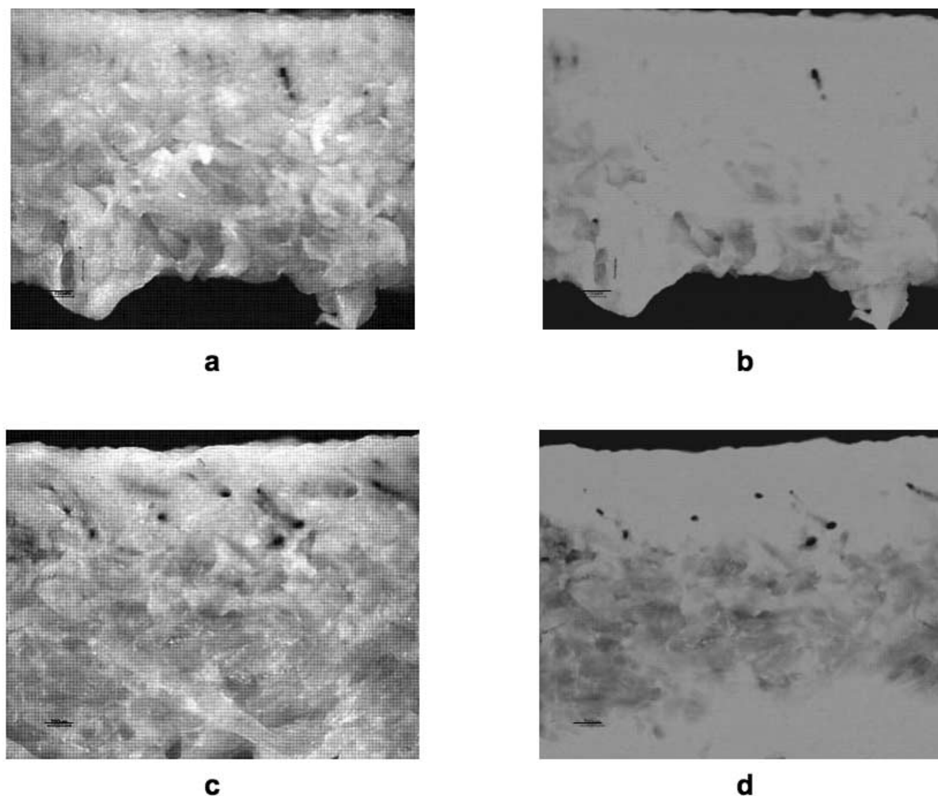


Figure 6. - Epi-fluorescent microscopic images of blue stock treated with a polymer prepared from gelatin, WPI and mTgase (offer 5% gelatin, 1% WPI based on weight of hide); (a and c) 519-nm emission from labeled gelatin, (b and d) 603-nm emission from labeled WPI. ( - = 750 μm).

The floats were drained and the labeled protein biopolymer solutions were added; the samples were tumbled for 1 h at ambient temperature, again to distribute the protein uniformly within the hide; and then for 4 h at 50°C, which is optimal temperature for enzyme reaction. The pH was then adjusted to below 4.0 using acetic acid (this pH will inactivate the enzyme); the floats were drained and the samples were washed twice (to remove any unattached protein) at 50°C. The samples, covered to prevent exposure to light, were stored at 4°C until examined.

**Epi-Fluorescent microscopic examination**

Since a fluorescent label was attached to the various proteins before polymerization, each of the treated products was examined for biopolymer penetration using a Nikon polarizing microscope, operating in optical mode, and equipped with an epi-fluorescent attachment. Samples were cut (grain to flesh) so that one could monitor depth of filler penetration and determine whether the filler was retained even after several washings. The control samples (blue stock to which no biopolymer or enzyme has been added, only pH-adjusting agents) shown in Figures 3a and 3b, were examined using the two different cubes (3a is control for Alexa Fluor 488® conjugate and 3b is control for Alexa Fluor® 568 conjugate). The light grey images are showing the fibers in the hide and, at the same time, that the blue stock auto-fluoresces. We observed this auto fluorescing phenomenon in a previous study when samples were examined using the confocal scanning laser microscope[2].

Figures 4a and 4b show the cross-section of hide that had been treated with the labeled polymerized gelatin and one can observe that the Alexa Fluor® 488 label attached to the gelatin is fluorescing. One can see that the product has penetrated the hide and that the distribution is uniform. This blue stock (Figure 4) looks almost amorphous compared to the control (Figure 3), in which one can still see the fibrous nature of the sample.

In Figure 5, two sections of blue stock that had been treated with the gelatin/casein biopolymer (5% gelatin and 1% sodium caseinate) are shown. Figures 5a and 5b show a section in which the biopolymer has fully penetrated (5a and 5c show emission at 519 nm and 5b and 5d show it at 603 nm). Figures 5c and 5d show treated blue stock, originally sampled from a denser area of the hide (i.e., butt area), and the biopolymer appears not to have penetrated evenly into the densely fibrous structure.

Figure 6 is showing the micrographs of blue stock that had been treated with a biopolymer made from enzyme-modified gelatin and WPI. Two sections were examined and Figures 6a and 6c show the labeled gelatin fluorescing and Figures 6b and 6d show the emission from the labeled WPI. In this experiment, 5% gelatin and 1% WPI were offered. Again these two sections are showing the extremes in fiber structure. Figures 6a and 6b show the sample in which the biopolymer has penetrated completely through the leather, while Figures 6c and 6d show a section which from the more densely structured butt area and the filler has penetrated but not to the extent one can see in the more loosely structured belly or neck area.

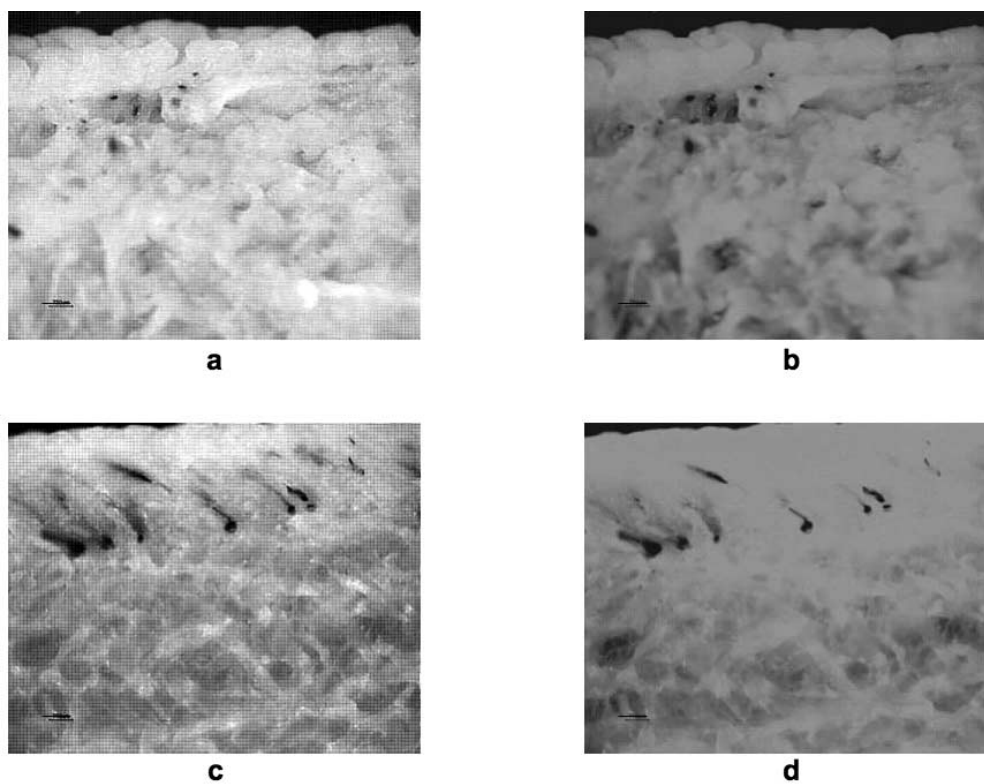


Figure 7. - Epi-fluorescent microscopic images of blue stock treated with a polymer prepared from gelatin, WPI and mTgase (offer 10% gelatin, 2% WPI based on weight of hide); (a and c) 519-nm emission from labeled gelatin, (b and d) 603-nm emission from labeled WPI. ( - = 750 μm).

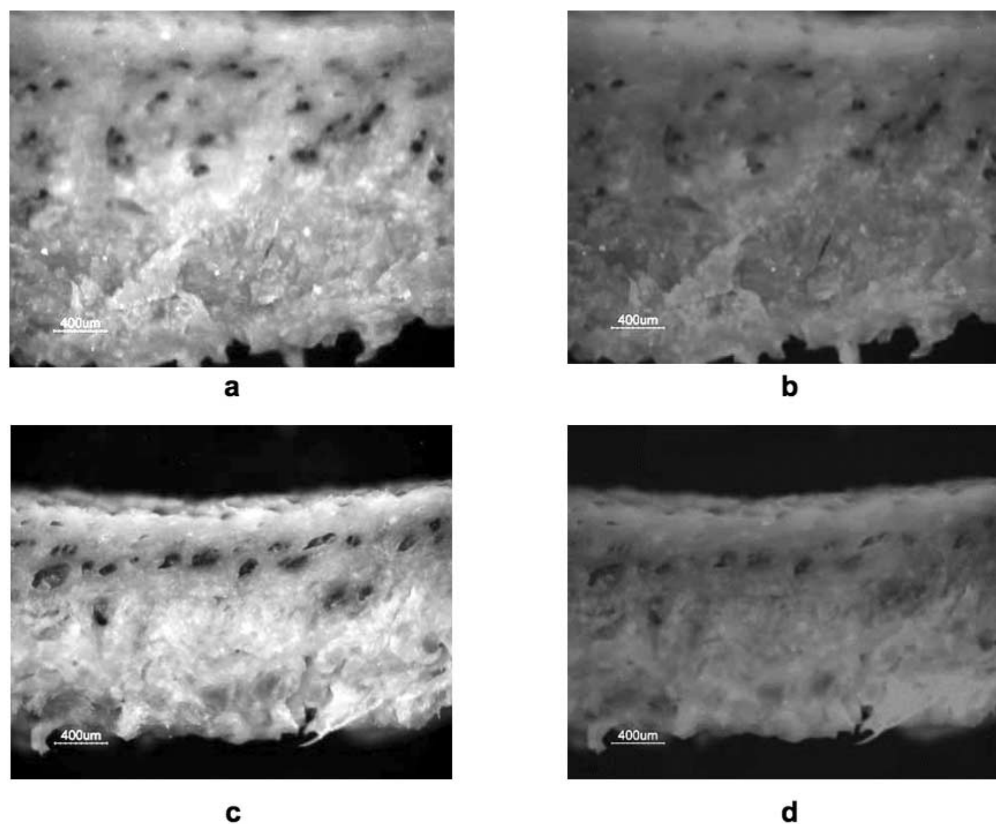


Figure 8. - Epi-fluorescent microscopic images of blue stock treated with a polymer prepared from gelatin, whey and mTgase (offer 10% gelatin, 2% whey based on weight of hide); (a and c) 519-nm emission from labeled gelatin, (b and d) 603-nm emission from labeled whey. ( - = 400 μm).

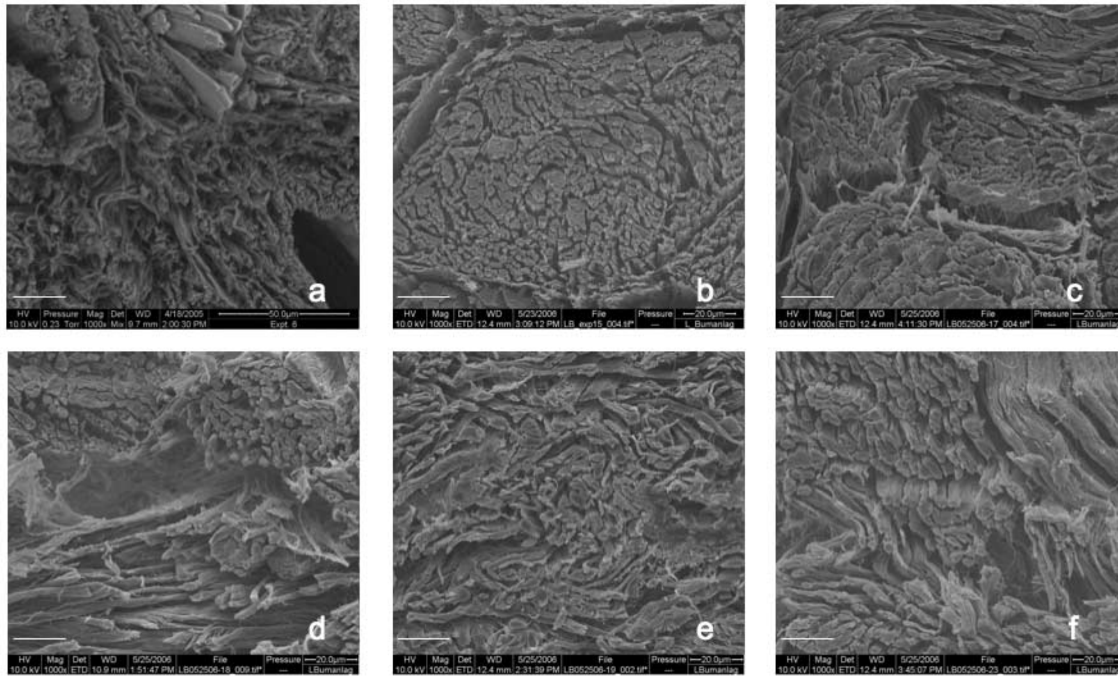


Figure 9. - Scanning Electron Microscope images of blue stock treated with pH-adjusting agents: no enzyme or gelatin (9a), enzyme and polymerized gelatin (5% offered) (9b), enzyme and polymerized gelatin/sodium caseinate (5%/1% offered) (9c) enzyme and polymerized gelatin/WPI (5%/1% offered) (9d) enzyme and polymerized gelatin/WPI (10%/2% offered) (9e) and enzyme and polymerized gelatin/whey (10%/2% offered (9f). ( - = 20 µm).

In the next experiment, we doubled the amount of gelatin and WPI offered to see if there would be an increase in filler uptake and the results are shown in Figure 7. The fluorescence emitted by the gelatin labels is shown in Figures 7a and 7c and that from the WPI label, in Figures 7b and 7d. The penetration of the biopolymer appears to be similar to that seen in Figure 6, where only half the amount of filler was offered.

In the final set of experiments, we evaluated the potential of making a filler product from the combination of gelatin and whole whey (with sugar). Figures 8a and 8b show a section of blue stock treated with 10% gelatin and 2% whey. Again we are seeing the amorphous type structure that is indicative of biopolymer penetration. Figures 8c and 8d show treated samples from a hide that was split more thinly than the above samples. In both cases, the product has penetrated fully.

**Scanning electron microscope examination**

The samples that had been examined using the epi-fluorescence equipped Nikon polarizing microscope, operating in optical mode, were also studied using the Scanning Electron Microscope. The samples were mounted on an aluminum probe and were examined at different magnifications. The 1000x magnification was chosen for the samples shown in Figure 9. In Figure 9a, blue stock to which no gelatin or enzyme were added, only sodium bicarbonate and acetic acid for pH adjustment, is shown. One can see that the fiber structure is compact. In Figures 9b through 9f we are showing blue stock, after pH adjustment, that was treated with enzyme and then with either polymerized gelatin (9b), gelatin/casein (9c), gelatin/WPI (9d and 9e) or gelatin/whey (9f). It appears that the samples treated with the polymerized products, as compared to the control (9a), have more regularity to their structure and the fibers appear to be larger in diameter. The fibers may be coated with the polymers and this observable fact might manifest itself in a filling effect in looser or veiny areas of the hides.

## Conclusions

Biopolymers were produced from the enzymatic treatment of fluorescently labeled gelatin and casein, WPI, or whey. These products were characterized and the gelatin/casein combination indicated a dramatic increase in gel strength whereas there was no significant change in that of the gelatin/whey combinations over the controls; the melting points and viscosities of all modified samples were increased. SDS-PAGE showed that molecular weight distributions were altered and higher molecular weight polymers were formed. When these biopolymers were applied to blue stock in filler experiments, and the samples were examined using the epi-fluorescent microscope, the results showed that the proteins were evenly distributed throughout a loose-type hide, but diffusion was restricted in the more dense area of the hide, such as in the butt area. More importantly, when examining the edges of the hides, the presence of fluorescence indicated that the biopolymer was not removed by washing. When the samples were examined using SEM, it appears that the treated samples, as compared to the control, have more regularity to their structure with the fibers appearing to be larger in diameter, which may be indicating that fibers may be coated with the polymer. Thus these reactions between dissimilar proteins (from renewable resources) have the potential to be used in leather processing as economical replacements to petroleum-based fillers and potentially as coatings.

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