Effect of Chromium in Blue Hides on Activity of Microbial Transglutaminase

by

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Abstract

Microbial transglutaminase (mTgase) has been used in the modification of proteins from sustainable resources to make products that have application in leather production. It has also been successfully used to treat blue stock, prior to addition of the products, for the purpose of stabilizing the fillers and lessening the chance that they will be washed out during further processing steps. There is always a concern that metals may have an effect (either positively or negatively) on the enzyme's activity and literature has addressed many of these issues. However, one area that has not been addressed is whether chromium may affect activity of mTgase. Even though we have only seen positive results after hide treatment with enzyme, we designed an experiment to elucidate this matter. Using atomic absorption spectroscopy, we first determined the concentration of chromium in the floats during the processing of the hides. When the hides were washed and then neutralized, the concentration of unbound chromium was found to be less than 5 ppm. Next, to test mTgase sensitivity, we carried out activity assays in absence and presence of chromium and found that activity results were similar. We prepared a model system in which gelatin, swollen and solubilized in solutions containing chromium, was reacted with mTgase using standard conditions. It was found, when compared to a control, that the reaction proceeded at the same rate (chemical gel formed in 2 h @ 50°C), and the physical properties and molecular weight distributions (SDS-PAGE) of the products were similar. Finally, epi-fluorescent microscopy studies showed that enzyme-pretreatment, when compared to a control in which no enzyme pretreatment was applied, prevented migration of the fillers during subsequent processing steps. Thus, under the conditions in which we treat wet blue hides, the chromium used to tan the hides does not affect enzyme activity.

Resumen

La transglutaminasa microbiana (mTgase) ha sido utilizada en la modificación de las proteínas procedentes de fuentes sostenibles para productos que tienen aplicación en la producción de cuero. También se ha utilizado con éxito para tratar inventarios de wet blues, antes de la adición de productos rellenantes, con el fin de estabilizarlos y disminuir las probabilidades de que se removieran por lavados durante los siguientes pasos del proceso. Siempre hay una preocupación de que los metales pueden tener un efecto (ya sea positivo o negativo) sobre la actividad de la enzima y la literatura ha abordado muchos de estos temas. Sin embargo, un área que no se ha abordado es si el cromo puede afectar a la actividad de mTgase. Aunque sólo hemos visto resultados positivos después del tratamiento de las pieles con enzimas, hemos diseñado un experimento para dilucidar esta pregunta. Usando espectroscopia de absorción atómica, se determinó por primera vez la concentración de cromo en la flota durante el procesamiento de las pieles. Cuando las pieles se lavan y luego son neutralizadas, la concentración de cromo no fijado fue encontrada menor a 5ppm. A continuación, para poner a prueba la sensibilidad mTgase, llevamos a cabo ensayos de actividad en ausencia y presencia de cromo y encontramos que los resultados de la actividad fueron similares. Hemos preparado un sistema modelo en el que la gelatina, turgente y disuelta en soluciones que contienen cromo, se reaccionó con mTgase bajo condiciones normales. Se constató, en comparación con un control, que la reacción procedió a la misma velocidad (gel químico formado en 2 horas a 50° C), y las propiedades físicas y la distribución del peso molecular (SDS-PAGE) de los productos son similares. Por último, estudios de microscopía epifluorescente mostraron que la enzima pre-tratada, en comparación con un control en el que no se aplicó el tratamiento previo de la enzima, impide la migración de los rellenantes durante los pasos de procesamiento posterior. Así, en las condiciones en que tratamos a los cueros wet blue, el cromo utilizado para curtir las pieles no afecta a la actividad enzimática.

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INTRODUCTION

Microbial transglutaminase (mTgase) has been used in the modification of proteins from sustainable resources to make products that have application in leather production.[1-3] It has also been used to treat the blue stock prior to addition of the products, for the purpose of stabilizing the fillers and lessening the chance that they will be washed out during further processing as demonstrated by our epi-fluorescent microscopy studies of mTgase treated and untreated hides.[4-6] There is always a concern that metals may have an effect (either positively or negatively) on the enzyme's activity and literature has addressed the influence of many metals on mTgase activity;[7-8] however, one area that has not been addressed is whether chromium, and in particular the chromium used to tan hides, may affect enzyme activity. We designed an experiment to investigate this matter.

In this study we first used atomic absorption spectroscopy (AAS) to determine the concentration of chromium remaining in floats after blue stock was washed, neutralized, enzyme pretreated, and the product applied. Knowing the concentrations of chromium in the reaction systems, we next determined if these concentrations of chromium would have, either positively or negatively, an effect on mTgase activity. Then, using a model system consisting of gelatin in the presence and absence of chromium, we reacted mTgase with gelatin under the appropriate conditions (pH, temperature, time, and concentration). The physical properties (gel strength, melting point and viscosity) and molecular weight distribution by SDS-PAGE, of the resultant products were examined and compared to controls (no enzyme and enzyme in the absence of chromium). Finally, blue stock was treated with varying concentrations of enzyme and then with fluorescently labeled modified fillers; the hides were examined for filler retention using epi-fluorescent microscopy. Chromium analyses, enzyme assays, physical properties, SDS-PAGE gels, and epi-fluorescent microscopy images will be presented.

EXPERIMENTAL

Materials:
Activa TG-TI, a microbial transglutaminase (mTgase) (approximately 100 units/g of product) 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). Whey protein isolate, WPI (Alacen™ 895) containing 93.2% protein, was 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). Chromosal B was obtained from Lanxess Corp. (Pittsburgh, PA). Chrome tanned stock, upholstery weight and shoe upper weight, were purchased from several area tanneries. All other chemicals were analytical grade and used as received.

Methods:
Treatment of blue stock for chromium analyses of floats
As described in a previous publication,[2] blue stock (shoe upper weight) was treated with mTgase and fillers as follows: a control treatment to which no enzyme or product was added, a treatment in which only the protein products were added (Test #1) and finally a treatment in which both enzyme and protein products were added (Test #2), were run (Figure 1). Aliquots of all floats were taken and set aside for atomic absorption spectrophotometry (AAS) analysis for chromium.

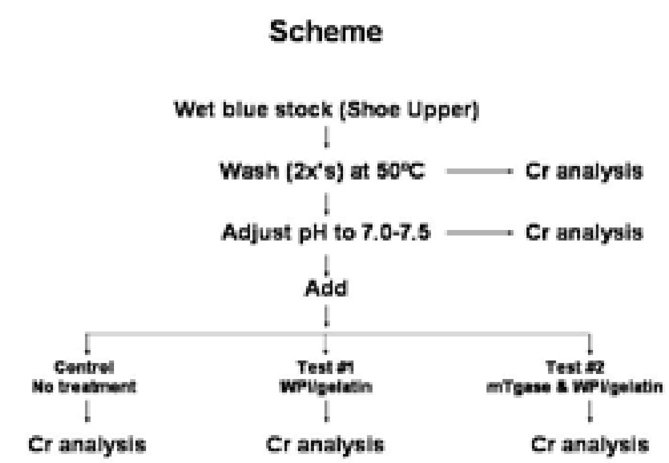

Figure 1. Flow diagram for treatment of blue stock and protocol for chromium sampling.

Treatment of gelatin with enzyme
Gelatin samples (175 g Bloom) were suspended in nano pure water or in 35 or 70 ppm chromium solutions (Chromasol B), allowed to swell for about 4 h and then placed in a bath at 65ºC until dissolved. The pH was adjusted to 6.5 with either 1N NaOH or 1N HCl. Control samples, to which no enzyme was added, and samples to which enzyme but no chromium was added, were run to monitor changes in physical properties. MTgase, (calculated to be 1 unit/g of gelatin), was prepared in 10 ml of water and this solution was added with stirring to the appropriate gelatin solutions to give a final protein concentration of 10% w/w. Ten-ml aliquots of the reaction mixtures were added to test tubes for melting point determination. The remainder of each solution was poured into appropriate containers for determining gel strength. The samples were warmed to 50ºC in a shaker bath and the reaction was carried out for 4 h. The enzyme was inactivated by heating the reaction products at 90ºC for 10 min. The samples were cooled to room temperature and then chilled for 17 hours at 10ºC 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.

Treatment of blue stock for epi fluorescent microscopy studies
a. Preparation of fluorescently labeled conjugates
Fluorescently labeled conjugates of gelatin and WPI were prepared using Alexa Fluor® 488 (absorbance at 494 nm and fluorescence emission at 519 nm) and Alexa Fluor® 568 (excitation at 577 nm and fluorescence emission maxima at 603 nm) protein labeling kits, respectively, as described in previous publications.[4-6] The labeled proteins were 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.

b. Preparation of fluorescently labeled polymer products
Gelatin samples in combination with WPI were suspended in water 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 WPI were added to the 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. The pH was adjusted to 6.5-7.0 with 1 N NaOH, 0.5% DTT (w/w, based on WPI) was added, and the samples were heated at 38°C for 1 h. MTgase (1 U/g gelatin and WPI) was 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% WPI. Aliquots (10 ml) of the reaction mixture were added to test tubes for melting point determination and a sufficient amount of each solution (30 ml) was poured into an appropriate container for determining gel strength. All 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 90ºC for 10 min. The samples were cooled to room temperature and then chilled for 17 h at 10ºC in a constant temperature bath. Physical analyses (gel strength, melting point and viscosity) were run and these results are shown in Figure 2. Aliquots of the samples were lyophilized and molecular weight distribution was determined (Figure 2).

Sodium azide (70 µl of 1% solution) was added as a preservative to the solutions to be used in wet blue treatment and these samples were stored, tightly covered, at 4°C until use.

c. Application of labeled filler to wet blue upholstery weight leather
Upholstery weight 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 (0, 3 or 4% based on wet weight of hide) was added, and samples were tumbled for 1 h at ambient temperature. The float was drained and labeled biopolymers (diluted to give a 400% float based on wet hide weight) were added to the drums. The samples were 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.

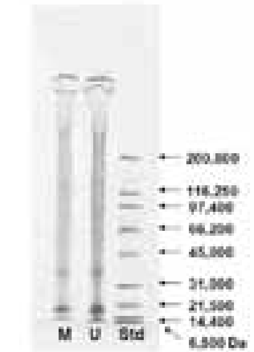

TABLE 1
Physical properties of gelatin/WPI biopolymers

Sample[a]	Gel Strength (g)	Melting Point (°C)	Viscosity @60°C (cP)
GelWPI U[b]	340.5	36.4	7.6
GelWPR M[cd]	330.5	36.8	7.9

[a]10% gelatin/2% WPI @ pH=6.5, Temp=50C, 4h;
[b]0 units mTgase; [c]1 unit mTgase; [d]N=3

Figure 2. SDS-PAGE of 175 Bloom gelatin, 10% w/w concentration and 2% WPI, untreated (U) and modified with 1 unit (per gm of protein) mTgase (M); molecular weights are shown in Da. (Table I, which contains physical properties of products).

Analyses:
Measurement of mTgase activity
MTgase activity was determined by the method of Folk and Cole,[9] with a modification by Cui et al.[8] The assays were run on mTgase that had been incubated at 37°C for 30 min with 0.2 mM chromium sulfate, the tanning agent used for bluing of hides.

Physical properties, molecular weight distribution and chromium analysis
Gel strength, melting point, viscosity, and molecular weight distribution (by SDS-PAGE) of the enzyme-treated proteins solutions were determined as described in previous publications.[10,11] Chromium was determined, as described previously.[12] using a Perkin Elmer atomic absorption spectrophotometer, Model 3300 (Norwalk, CT) equipped with WinLab™ software.

Epi-fluorescent microscopy
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[13] and with a digital camera (DXM 1200).

Results and Discussion

Chromium analyses of treatment floats

To understand the magnitude of the chromium present during the various filler treatments, we designed an experiment in which all floats during the blue stock treatment were sampled. Three different processes were run (Figure 1): one in which no enzyme or filler was added, a second in which no enzyme, only filler, was added, and lastly, one in which the blue stock was pretreated with mTgase before addition of the filler product.

All floats were sampled (Figure 1) and the results of the chromium analyses are shown in Figure 3. The standard deviations for the analyses (as indicated by the error bars) ranged from an average of 0.40 for the wash #1 floats down to 0.03 for the floats sampled after 5 hours.

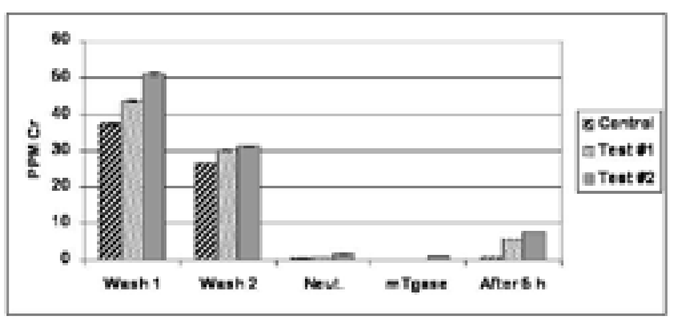

Figure 3. Chromium analysis, expressed in ppm (Std Dev is indicated by error bars), from blue stock treated with pH adjusting agents (control), with WPI and gelatin (Test #1) and with mTgase and then with WPI and gelatin (Test #2).

The data indicate that the highest chromium concentration can be found, as one would expect, after the first wash (51 ppm or less). After the second wash the concentration is about half and after pH adjustment the chromium concentration had dropped to less than 2 ppm. If an enzyme pretreatment is carried out, the chromium present in the resulting float is also quite low (1 ppm). Even after the filler treatment, the chromium concentration is less than 1 ppm to about 8 ppm, indicating that no significant amount of chromium is present in the float. This experiment has defined the amount of unbound chromium that would be present when the enzyme reaction is run. Obviously the washes would remove most of the unbound chromium before the enzyme would be added.

Enzyme activity in presence of chromium

Based on the information as to how much chromium the enzyme would be exposed to in a typical reaction, as described above, we designed an experiment in which the mTgase assay was run in the presence of approximately 8 ppm chromium. The results can be seen in Figure 4 and show that when the assay was run in the usual method (in water) the activity was 98.6 U/ mg enzyme and when run in chromium solution, 97.7 U/ mg enzyme. The results are not significantly different (standard deviation as shown by the error bars) and indicate that at this concentration of chromium there is no influence on enzyme activity.

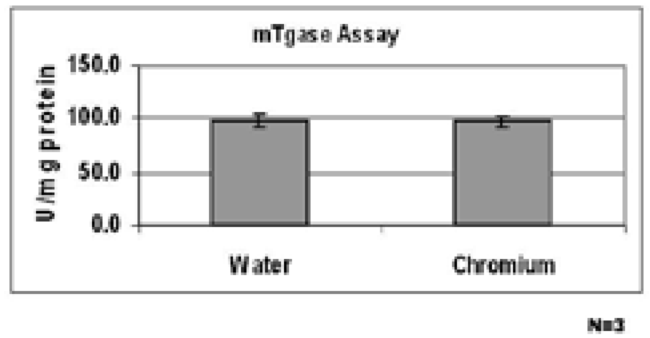

Figure 4. Assay of mTgase in water and in an 8 ppm Cr solution. Activity is expressed in units per mg of protein (Std Dev is indicated by error bars).

Reaction of gelatin with mTgase in presence of chromium

Based on prior research,[10-11] we have used a 10% w/w concentration of gelatin treated with 10 units of mTgase to do an approximate evaluation of enzyme viability. Typically, these reactions proceed quite rapidly, giving a chemical gel in about 2 h at 50°C and pH of 6.5-7. A chemical gel is one that, unlike unmodified gelatin gels, will not return to a liquid state upon heating.

We designed an experiment in which a model system using gelatin was reacted with mTgase in the presence and absence of chromium. The chromium reaction solutions contained 35 and 70 ppm respectively and the chromium in these solutions was prepared from a commercial chrome tanning agent. As was shown in the data above, the amount of chromium in these model systems is much higher than one would find after washing and pH adjustment of the hides prior to filler treatment.

To demonstrate whether the enzyme would or would not be inactivated in the presence of chromium, four reactions were carried out, each in duplicate. When the reactions were complete, physical properties (gel strength, melting point, and viscosity) and molecular weight determinations were performed. The results of the physical property analysis can be seen in Table II.

A control, to which no enzyme was added, had a gel strength of 427 g, melting point of 35.2°C, and viscosity of 5.5 cP @ 60°C. The properties of a secondary control, a sample in which the gelatin was swollen in nano-pure water and to which mTgase was added, are shown and the gel strength is 525 g, but the melting point is greater than 90°C and, because of the solid gel, the viscosity cannot be determined. These data are typical of those

TABLE II

Physical properties mTgase-treated gelatin[a]

Sample	Gel Strength[b] (g)	MP(°C)	Viscosity (cP @ 60°C)
Control	427	35.2	5.5
Control w enz	513	>90	na[c]
Test low 35 ppm Cr	486	>90	na[c]
Test high 70 ppm Cr	478	>90	na[c]

[a]10% w/w, pH=6.5, temp. = 50°C, 4 h, [b]Aver STDEV = 20;
[c]na=solid gel, could not be determined

we have seen in prior experiments, in which a chemical gel has formed when the appropriate conditions are applied. Finally in those gelatin samples swollen in the chromium solutions (35 ppm and 70 ppm), the gel strength value of the 35 ppm sample is not significantly different than the secondary control sample but that of the 70 ppm sample is a little lower; in both cases, chemical gels have formed, the melting point is greater than 90°C and, because of the solid gel, viscosity cannot be determined. These physical properties are indicating that the chromium did not significantly interfere with the mTgase activity.

To further corroborate that the enzyme's activity was not affected, we ran SDS-PAGE gels to determine molecular weight distribution of the products, and these results are are shown in Figure 5. One can observe that the bands typical of gelatin (6,500-200,000 Da) can be seen in the control sample (Lanes 2 and 3). In the secondary control, containing mTgase and no chromium (Lanes 4 and 5), these bands have almost disappeared, including the band that does not enter the gel (> 200,000 Da).

This is consistent with the production of a polymer too large to be solubilized by the SDS sample buffer used to prepare materials for SDS gel electrophoresis. In the two samples that contained the chromium (Lanes 6 and 8, 35 ppm Cr; Lanes 9 and 10, 70 ppm Cr), the results are identical to the secondary control in that the typical gelatin bands are no longer visible. The data show that the enzyme retained its activity even in the presence of chromium in excess of what is typically present in our processes and indicate that unbound chromium should not interfere during the enzyme pretreatment of blue stock and the subsequent filler reaction.

Epi-fluorescent microscopy studies of treated blue stock.

In theory, the chromium that has been incorporated into the tanned hide could be referred to as being "bound" and should have no effect on the enzyme reaction. However, to further substantiate as to whether this "bound" chromium may or may not interfere with mTgase activity during the filler treatments, we designed an experiment to show the efficacy of enzyme

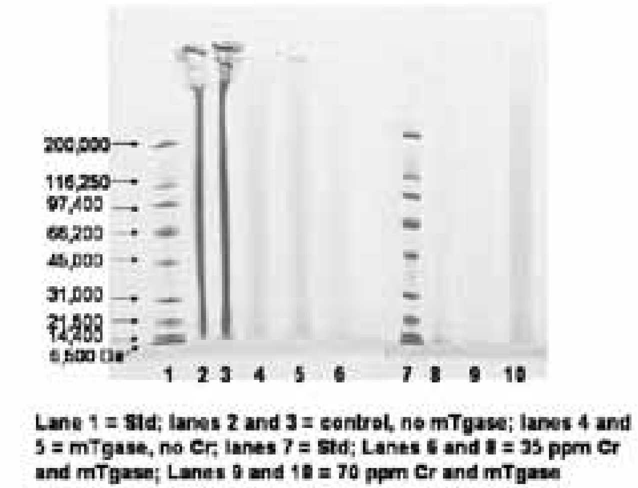

Figure 5. SDS-PAGE of 175 Bloom gelatin, 10% w/w concentration, pH 6.5, temperature 50°C, 4 h; molecular weights are shown in Da (equivalent sample weights apply to each lane).

pretreatment of blue stock. This treatment insures that the fillers do not migrate out of the hides during washing or subsequent retan, color and fatliquoring steps. To treat the blue stock (upholstery weight), a biopolymer was prepared and characterized as described in the Experimental section. The blue stock was first pretreated with varying concentrations of mTgase (0, 3, 4%) and then with 10% gelatin/2% WPI biopolymer product (Figure 6).

Upon completion of the treatment, the treated blue stock was washed twice with water at 50°C for 10 min and then samples were taken from both the edges and inside of the treated hides for microscopic examination. These samples were examined using the epi-fluorescent microscope at both 519 nm and 603 nm. As has been seen previously,[4-6] blue stock auto-fluoresces at these wave lengths, however, when the labeled products are present the images take on an amorphous character and fiber structure is difficult to see. Representative images (emission at 519 nm) of these samples are shown in Figure 7. At the 0% mTgase offering, fiber structure can still be seen, indicating little or no filler present. At the 3% offering, the fibers are less distinct and at

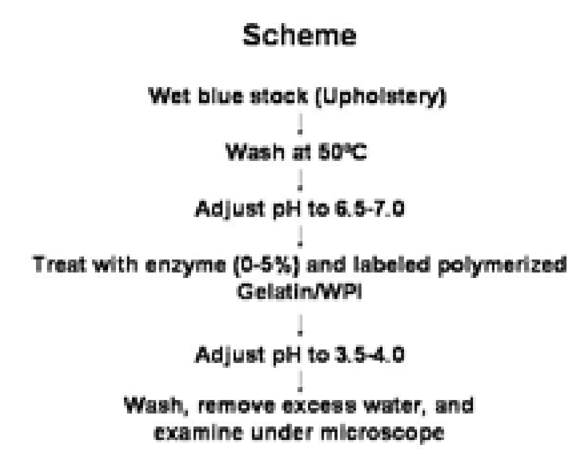

Figure 6. Schematic for treatment of blue stock with labeled gelatin/WPI biopolymer product.