Studies on the Removal of Inter-fibrillary Materials Part I: Removal of Protein, Proteoglycan, Glycosoaminoglycans from Conventional Beamhouse Process

by

B. Madhan, J. Raghava Rao* and Balachandran Unni Nair
Central Leather Research Institute
Council of Scientific and Industrial Research,
Adyar, Chennai-600020, India

Abstract

The removal of the inter-fibrillar materials viz., proteins, proteoglycans and glycosoaminoglycans (GAGs) are the integral part of conventional liming and bating processes. However, precise quantification on the removal of these inter-fibrillar materials is still lacking. Hence, in the present work quantification on the removal of protein, proteoglycans and glycosaminoglycans (from goat skins) during liming and bating was attempted. An understanding of the removal of these inter-fibrillar materials precisely could help in developing objective process control measures. Alternative techniques can be developed as it is known that conventional liming processes contribute to more than 60% of the total pollution load. The quantification of proteins, proteoglycans and glycosoaminoglycans was standardized to suit the requirement for the estimation of the samples obtained from beamhouse process liquors (from liming to bating). It is observed that 3.2% proteins, 1.2% proteoglycans and 0.04% GAGs are released (based on wet salted weight) in the pretanning processes, from liming to bating. Removal of proteoglycans during liming has been found to be dependent on the duration of liming process.

Resumen

La remoción de los materiales interfibrilares, es decir, proteínas, proteoglicanos y glicosaminoglicanos (GAGs) son parte integral de procesos de pelambre y rendido convencionales. Sin embargo, cuantificación precisa sobre la remoción de tales materiales interfibrilares todavía no existe. Por esta razón, en el presente trabajo cuantificando la remoción de proteínas, proteoglicanos y glicosaminoglicanos (de pieles caprinas) durante el pelambre y rendido se trato de efectuar. Una comprensión acerca de la remoción de estos materiales interfibrilares podría ayudar precisamente en el desarrollo de que medidas tomar para los procesos de un control objetivo. Técnicas alternas podrían desarrollarse ya que es conocido que el 60% de la total carga contaminante se origina en el pelambre convencional. La cuantificación de proteínas, proteoglicanos y glicosaminoglicanos fue estandarizada a la medida del requisito para la estimación de muestras obtenidas de los efluentes de los procesos de pelambre (desde el encalado através del rendido). Es observable que 3,2% de proteínas, 1,2% de proteoglicanos y 0,04 de GAGs son liberados (en base a peso salado en húmedo) en los procesos previos al curtido, desde el pelambre al rendido. La remoción de proteoglicanos durante el pelambre se ha encontrado dependiente de la duración del proceso de pelambre.

*Corresponding author e-mail: clrichem@mailcity.com
Manuscript received August 4, 2009, accepted for publication January 19, 2010

Introduction

Pretanning or beamhouse processes/operations from soaking to bating adopted for leather processing primarily aims at the purification of collagen, the leather making protein. Liming is one of the important pre tanning process and its objectives are removal of hair, flesh and separation of the fibre bundles physico-chemically.[1] Hair can be either digested or mechanically removed after loosening by chemical means (conventional process). The pH of the pelt is increased to 12-13 by the use of lime, which results in osmotic swelling due to ionic imbalances built up in the matrix.[2] The removal of flesh is actually facilitated by the action of hydrostatic pressure and then subsequently removed using a shearing force provided by a fleshing machine or knife. Commercially, several methods of application are being adopted for liming viz., pit liming, paddle liming, drum liming, paint liming etc., depending on the requirement and facility available in the tannery.[3] The method for the removal of hair could be selected based on several factors. For example, in pit, paddle and drum liming, hair is damaged since the depilating agents attack directly the cystine in hair shaft as well as in hair bulb.[4] In the paint method, the depilating agent, applied on flesh side diffuses through the matrix and attacks the cystine in hair bulb to cause hair loosening leading to hair saving.[5,6] Enzymatic unhairing (using enzymes along with small amounts of sodium sulphide and lime applied as paint on flesh side) causes loosening of hair by selective breakdown of cementing substances and presents a hair saving approach.[7-9]

Apart from liming, the other important process in pre tanning is bating, which aims at the removal of some of the inter-fibrillar materials mainly proteins and proteoglycans. The removal of these inter-fibrillar materials is facilitated by the use of proteolytic enzymes in bating. The removal of inter-fibrillar materials viz., proteins, proteoglycan and glycosaminoglycans during liming and bating process is a well established fact. However, till date the quantification of these materials had not been known. Hence, in the present work quantification on the removal of protein, proteoglycans and glycosaminoglycans (from goat skins) during liming and bating has been carried out. Such quantification not only improves our understanding on beamhouse processes but also facilitate the development of alternative technologies for cleaner and greener processing.

Experimental

Materials

Wet salted goat skins (5-6 sq. ft.) were chosen as raw materials for the study. All chemicals used for leather processing were of commercial grade while the chemicals used for the analysis of spent liquors were of analytical grade. Bovine serum albumin (BSA), Chondroitin sulphate, Mucin, Folin ciocelatu reagent, Periodic acid and Iodo acetic acid were procured from Sigma-Aldrich Chemicals, India and other analytical chemicals were procured from SRL Chemicals, India.

Quantification on the Release of Protein, Proteoglycan and GAGs in Conventional *Processes*

Four wet salted goat skins were taken and processed using the conventional leather process. Liming process was carried out for 4 days and sample liquor from each day of liming and subsequent process liquors viz., lime wash liquor, delime liquor, bate liquor and bate wash liquor were also collected. Volume of the respective process liquors was measured. The pH of the lime liquor alone was brought down to ~ 8.0 before analysis. All the samples were filtered through Whattman filter paper. The filtered samples were taken for the estimation of protein, proteoglycan and GAGs spectrophotometrically using standard procedures.[10-14]

Estimation Methods

Lowry's method for protein estimation[10, 11]

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. Hydrolyzing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et al (1951)[12] is sensitive enough to give consistent value and hence largely followed. The reagents used are 2% sodium carbonate in 0.1N sodium hydroxide (Reagent-A), 0.5% copper sulphate ($CuSo_4.5H_2O$) in 1% of potassium sodium tartarate (Reagent-B). 50 ml of reagent A and 1ml of reagent-B is mixed prior to its use (Reagent-C), and Folin ciocalteau reagent (Reagent-D).

Working standard: 50 mg of BSA was dissolved in distilled water and made up to 50 ml (stock solution). Working standard (0.2 mg BSA/ml) was prepared by diluting 10 ml of the stock solution to 50 ml with distilled water in a standard flask.

BSA set of standards were prepared by pipetting out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes and made up the volume to 1 ml in all the test tubes. A tube with 1ml of water served as the blank. 5 ml of reagent-C was added to each tube, mixed well and allowed to stand for 10 minutes. Then 0.5 ml of reagent-D was added, mixed well and incubated for 30 min in the dark. All the steps are carried out at room temperature. Spectral reading at 660 nm using Cary 100 UV-Vis spectrophotometer of Varian was taken subsequent to the development of blue color. Standard curve was prepared by plotting the absorbance against the known BSA set of standards. The amount of protein present in the hide samples were extrapolated using the standard curve.

Proteoglycan estimation[13]

This Schiff's assay is useful for screening column fractions for periodate oxidizable glycoconjucates. The reagents used are, 1) Periodic acid. Just before use, mix 10 µl of the 50% of periodic acid with 10 ml of 7% acetic acid. 2) Schiff reagent. To the set of 2 to 50 µg of mucin (glycoprotein) standards in 1ml of water, 100 µl of the freshly prepared periodic acid solution was added, mixed and incubated at 37°C for 2 hr. For full color development, 100 µl of decolorized Schiff's reagent was added, mixed and allowed to remain at room temperature for 30 min. Absorbance at 555 nm was measured using Cary 100 UV-Vis spectrophotometer of Varian. Appropriate water or buffer blanks were used. The mucin standard curve was prepared by plotting the absorbance at 555nm against the series of mucin standard concentration. The amount of proteoglycan present in the samples were extrapolated from the curve or calculated using the slope of the mucin standard graph.

Glycosaminoglycans estimation — Modified dimethylmethylene blue assay[14]

The modified dimethylmethylene blue assay that has improved specificity for sulphated glycosaminoglycans and in conjuction with specific polysaccharidases was used to quantitate individual sulphated glycosaminoglycans. The color reagent was prepared by dissolving 16 mg dimethylmethylene blue in 1l water containing 3.04 g glycine, 2.37 g NaCl and 95 ml 0.1 M HCl, to give a solution at pH 3.0, with A_{525} 0.31. The reagent was stable in a brown bottle at room temp for at least 3 months. Color reaction: 100 μl of each sample, containing up to 5 μg of glycosaminoglycans, and 2.5 ml dimethylmethylene blue color reagent was added. Mixing was completed by pouring the solution into a disposable spectrophotometric cuvette, and A_{525} was read immediately using UV-Vis spectrophotometer. The assay was calibrated by use of reagent blanks and standards containing up to 5 μg Chondroitin sulphate. Papain digestion: Digestion of other interfering proteins was carried out using papain. Initially the sample in 1.0 ml containing 20mM sodium phosphate buffer (pH 6.8), 1mM EDTA, 2mM dithiothreitol and 300 μg papain, was incubated at 60°C for 60 min. The digested sample was made up to 5 ml by the addition of Iodoacetic acid 10 mM (final concentration) and 50 mM tris/HCl (pH 8.0). After this initial treatment, 100 μl of sample and 2.5 ml of dye were mixed as mentioned above.

RESULTS AND DISCUSSION

Standardization of Procedures for the Determination of Proteins, Proteoglycans and GAGs

Protein estimation

Skin matrix apart from collagen also contains other fibrillar and non-fibrillar proteins. The proteins under the non-fibrillar category viz., albumins, globulins etc. are expected to be removed during liming process as they are water and alkaline soluble. Lowry's method of protein assay was used to determine non-fibrillar proteins. Bovine serum albumin (BSA) was used to prepare the standard curve in determining the protein concentration. The standard graph was prepared by plotting the absorbance of increasing BSA concentrations (Figure 1) that were simultaneously done with different samples.

Estimation of proteoglycan

In order to determine the amount of proteoglycan released from the pre tanning process liquors, Schiff's proteoglycan assay was employed. Mucin was used as a standard proteoglycan in preparing the corresponding proteoglycan standard graph (Figure 2).

Glycasaminoglycans (GAGs) Standard graph

The chondroitin sulphate was used as a standard for the determination of GAGs in the process liquors of pretanning processes. The standard curve of the chondroitin sulphate is shown in Figure 3.

Removal of Protein, Proteoglycan and GAGs from Goat Skins Processed by Conventional Liming and Bating

Conventional beamhouse process, from soaking to pickling, was carried out using wet salted goat skins. The process liquors from liming to bating were analyzed for protein, proteoglycan and GAGs. All the process liquors were filtered prior to the analysis. In the measurement of protein, proteoglycan and GAGs in lime liquors, the pH was first adjusted to 8.0, since the absorbance (optical density) of the standard protein/proteoglycan/GAGs determined spectrophotometrically was found to vary at pH >11. The absorbance values of the process liquors pertaining to the concentration of the protein, proteoglycan and GAGs were calculated by using their respective standard graphs. The volume of process liquor was taken into account in the quantification and the values are reported as mg per gram of raw weight (Table 1). The raw weight refers to the weight of wet salted goat skins used for the study at moisture level of 30%.

It was observed that significant amount of proteins and proteoglycans were released into the lime liquors. About 25 mg of protein (per gram of wet salted weight) is being released from the lime liquor alone. Proteins like albumin and globulins are highly soluble in alkaline conditions and hence the release of high amount of protein during liming seems to be justified. The osmotic pressure exerted on the skin matrix during liming also facilitates the removal of proteoglycans. The amount of proteoglycan released is dependent on the duration of liming, which clearly seen from Figure 4. Washing the limed pelt prior to deliming and during deliming processes, significant amount of proteoglycan was being released into process liquor. The reason for such high amounts of proteoglycans being released during such processes could be that the inter-fibrillar

materials broken down (osmotic force) during liming and those physically adhering to the matrix were coming out into the liquor, largely facilitated by the mechanical agitation (drum). Again during bating around 1.5 mg of proteoglycan is removed by the activity of the proteases on the inter-fibrillar materials which is still remaining as remnants with the matrix even after earlier processes. Based on the raw weight of the skin matrix (goat) release of 3.2% proteins, 1.2% proteoglycans and

TABLE I

Protein, Proteoglycan and GAGs released from conventional beamhouse processes; from liming to bating

Process	Protein (mg/g of raw wt#)	Proteoglycans (mg/g of raw wt)	GAGs (mg/g of raw wt)
Liming liquor (day 1)	17.2±0.32	2.01±0.15	0.12±0.00
Liming liquor (day 2)	23.5±0.51	3.17±0.21	0.17±0.01
Liming liquor (day 3)	24.9±0.62	3.82±0.22	0.17±0.01
Liming liquor (day 4)	25.8±0.58	4.26±0.31	0.18±0.01
Wash liquor (10 min)	1.56±0.05	1.83±0.11	0.04±0.00
Delime liquor	2.95±0.07	2.87±0.12	0.05±0.01
Wash liquor (10 min)	0.84±0.01	0.70±0.03	0.04±0.01
Bate liquor	0.98±0.02	1.48±0.01	0.07±0.01
Bate wash liquor	0.34±0.004	0.52±0.03	0.02±0.00
Total *	32.47±1.18	11.66±0.61	0.40±0.06

- Raw wt refers to wet salted weight (30% moisture)
* - Cumulative addition from liming to bating

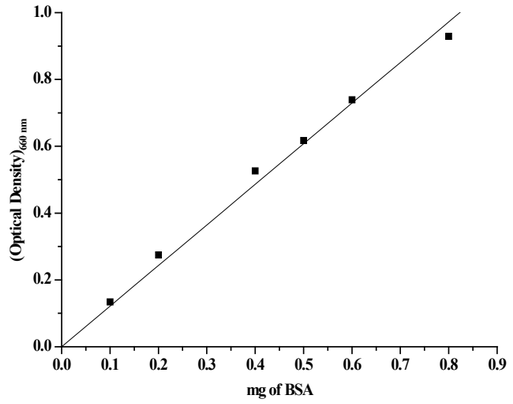

Figure 1. Standard graph for the determination of protein (Lowry's protein assay; using bovine serum albumin used as standard)

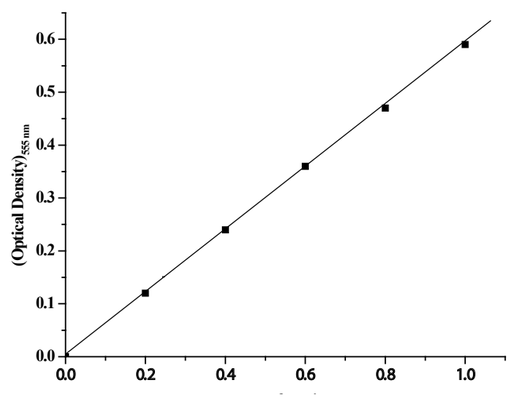

Figure 2. Standard graph for the determination of proteoglycan (Schiff's proteoglycan assay; mucin is used as standard)