Enzymatic Hydrolysis of Limed Trimmings: Preparation, Characterization and Application of Collagen Hydrolysate

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Abstract

Limed pelt trimmings are one of the solid wastes that are generated in leather processing. These wastes are valuable resources for producing collagen hydrolysate, which can be potentially used for retanning of leathers. This work establishes the preparation, characterization and application of collagen hydrolysate by enzymatic hydrolysis of limed trimmings. The collagen hydrolysate was prepared using various concentration of trypsin. The hydrolysate samples were characterized using Fast Protein Liquid Chromatography (FPLC) and Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) techniques. About 6 predominant fractions of collagen hydrolysate peptides were observed from the enzymatic hydrolysis. The molecular weight of collagen hydrolysates was observed to be in the range between 1750 and 5800 daltons. The collagen hydrolysates prepared using various concentration (0.8, 1.0 and 1.2%) of trypsin were used for retanning process. The collagen hydrolysate product obtained from hydrolysis with (0.8% trypsin, 3 hours) exhibited better dye uptake when used as retanning agent. Furthermore, collagen hydrolysate retanned leathers exhibited very good strength properties in comparison to leathers processed using control protein syntan. The option of internalizing the waste on one side and using them as a substitute for a high value product on the other presents the utilization of limed trimming as a strong case for sustainable leather manufacture.

Introduction

Sustained development in leather manufacture calls for research initiatives toward zero liquid and solid discharge. About 650 kg of solid wastes are generated from tanning industries per 1000 kg of wet salted raw hide/skin processed.[1] These solid wastes contain collagen protein, a valuable resource. Collagen has a wide range of application such as for making gelatin, additive component for cosmetics and as biomaterial for varied biomedical applications.

About 5-10% of the weight of hides are trimmed in beamhouse operation mainly for the ease of handling hides/skin in leather making. The practice of trimming the offal and other undesired portions of hides and skins is carried out before the soaking process. In Ethiopia, in addition to trimming in raw stage, the practice of trimming after liming had been very common, especially in the case of bovine hides. Nearly 2 million pieces of bovine hides with an average weight of 15 kg per piece are processed annually in the tanneries in Ethiopia. About 50-100 kg of limed trimming wastes are generated per 1000 kg of wet salted raw hide processed. So annually about 2000 metric tons of limed trimmings are generated from Ethiopian tanneries.

In our approaches to internalize this waste within the tanning industry, we explored the preparation of collagen hydrolysate syntans, a greener option amidst a wide variety of non biodegradable to extremely slow biodegradable syntan. We present an approach for making syntan from limed trimmings. Earlier reports on collagen hydrolysates were mostly based on raw trimmings or chrome shavings.[2,3,4] Kasparkova *et al.* reported the preparation of collagen hydrolysate using chemical hydrolysis.[5] Chen *et al*., and Taylor *et al.* have separated the protein from chrome shavings and have carried out the protein hydrolysis using enzyme.[6,7] Zhang *et al*. have attempted to make collagen hydrolysate from the gelatin extracted from limed splits.[8]

Enzymes such as collagenases have the ability to break down collagen in its native form. However, a wide range of proteases can act on the thermally denatured form of collagen including trypsin and pepsin.[9] In this work, we have strategized conditions of the limed trimmings amenable for trypsin treatment and characterized these collagen hydrolysates.

Protein syntans a being used in retanning applications, however their usage has been limited due to relative cost. Recently, in view of restrictions on the use of chemicals in leather manufacture there has been renewed interest in the use of such natural products. Karthikeyan *et al.*[10] have reported the use of

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keratin hydrolysate for retanning and they found it be very effective for filling application. Chen *et al*. and Taylor *et al*. have also reported on the use of collagen hydrolysates from chrome shavings in leather filling applications. In this paper, we present a novel and simple approach for the preparation of collagen hydrolysates from limed trimming waste and demonstrate the effectiveness of these collagen hydrolysate compositions as a retanning auxiliary in leather manufacture.

Experimental

Materials

Limed trimming wastes and wet blue leathers, from goatskins, were obtained from a tannery. Trypsin was procured from Sigma Aldrich chemicals. All other chemicals used for the analysis were of analytical grade and chemicals for processing leather were of commercial grade.

Methods

Preparation of Collagen Hydrolysate

Limed trimmings were collected and delimed completely with 100% water and 1% ammonium chloride based on the weight of trimmings. The delimed trimmings were cut into small pieces. 200 g of delimed trimmings were weighed and transferred in to a 1000 ml conical flask. Then 300% (v/w based on the wet weight of delimed trimmings) of water was added. The collagenous matrices in trimmings were denatured by heating (in water bath) at 80° C for 30 minutes. The hot melted dispersion was cooled to 37° C and trypsin (based on the weight of delimed trimmings) was added into the flask. The flask was then placed into shaker incubator and the speed of the shaker was adjusted to 200 rpm, temperature at 37°C and the hydrolysis was carried out for 3 hours. After that the flask was removed from the incubator shaker and placed in water bath and heated at 80° C for 5 minutes to deactivate trypsin enzyme and stop any further hydrolysis.

After cooling to room temperature, the extracted collagen hydrolysate was filtered off with Whatman filter paper. The residue on the filter paper was discarded and the liquid phase supernatant was collected and placed in deep freezer at -40°C overnight. Then it was lyophilized to get white collagen hydrolysate powder. Three experimental trials for the preparation of collagen hydrolysates *viz*., CH-1, CH-2, and CH-3 were carried out with varied amount of trypsin 0.8, 1.0 and 1.2% respectively.

Characterization of the Collagen Hydrolysate

Fast Protein Liquid Chromatography (FPLC)

15 mg/ml of collagen hydrolysate was prepared by dissolving collagen hydrolysate in distilled water, the mobile phase. The samples were filtered with sterilized MCE membrane filter of pore size 0.22µm. The column was packed by using 150 ml of Superdex 30 (prep grade) from GE Health care. Superdex 30, a preparative gel filtration medium with a composite matrix of dextran and agarose had been designed for purification of biomolecules such as peptides, oligosaccharides and small proteins in the molecular weight range up to 10 kDa. First the mobile phase alone was eluted for more than 2 hours in order to remove the ethanol, which has been used as preservative for the packing material. The flow of the mobile phase was set at 0.5 ml/min and the pressure was maintained at 1.0 MPa. After reaching equilibration of the mobile phase, 1 ml of the sample was injected in 0.5 ml loop; the excess volume of the sample gets discarded through an overflow bypass. All three collagen hydrolysate samples viz., CH-1, CH-2 and CH-3 were subjected to analysis using FPLC.

Matrix Assisted Laser Desorption/Ionization (MALDI-TOF)

In order to determine the molecular weight fraction of Collagen hydrolysate prepared by enzymatic hydrolysis, one of the samples CH-1 prepared using 0.8% of trypsin was subjected to MALDI-TOF analysis. The samples were analyzed for their molecular mass by using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectroscopy (Bruker Daltonik GmbH) where sinapinic acid (SA) was used as a matrix. The matrix was prepared by dissolving SA with 0.1% Trifluoro Acetic Acid (TFA) and acetonitrile. 1 µl of the prepared matrix (after centrifugation) was mixed with collagen hydrolysate sample and applied onto the MSP 96 polished steel microplate, kept for drying in ambient air and assessed for molecular mass. The spectrum of the hydrolysate m/z over a wide mass range versus intensity was plotted.

Application of Collagen Hydrolysate in Retanning

Goat wet blue leathers were sided in to four quadrants by cutting across the back bone and along the belly as shown in Figure 1. Quadrant I was used as a control and treated with commercial

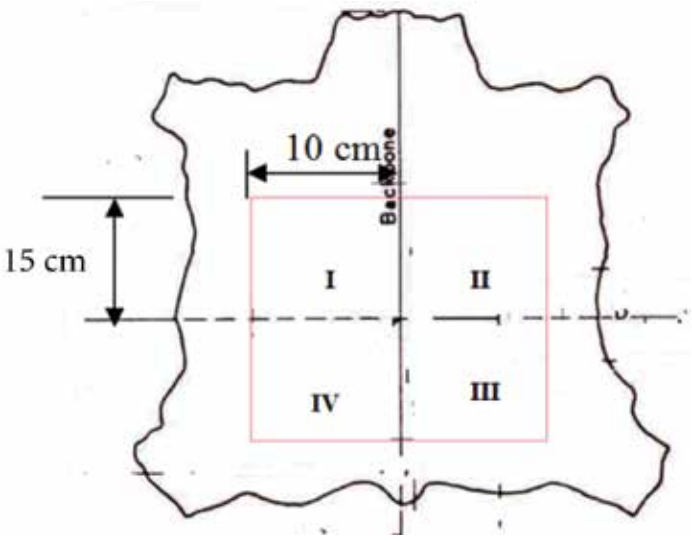

Figure 1. Allocation of the four quadrants of the wet blue goat leather used for determining the efficacy of collagen hydrolysate for retanning.

protein syntan for retanning; quadrant II was treated with the prepared collagen hydrolysate CH-1, quadrant III with CH-2, and quadrant IV with CH-3. The offer of the syntans in both the control and experiment were 10% based the shaved wet blue leather weight. No other syntans were used in both the control and experiment. Acrylic drums of the same size were used for processing control and experimental leathers, and the same post tanning process formulation was used except for the use of retanning agents.

Determining Percent Absorption of CH

The discharged post tanning liquor was collected and volume was determined. And then 3 ml of the sample was taken and evaporated in hot air oven. The dried matters were then dissolved in and made up into 10 ml volumetric flask with distilled water. From this stock solution, 200 µl was taken for hydroxyproline estimation according to the procedure of J. F. Wossener.[11]

Determining the Percent Absorption of Dye

Known concentration (5, 10, 20, 30, 40, 60, 80 and 100 ppm) of acid blue dye (used for post tanning) were prepared. The λmax for the dye was determined to be 610 nm. The absorbance of the known concentration was read at λmax.

The discharged post tanning liquor was collected and the total volume of spent liquor was determined. 1ml of the spent liquor was transferred into 10 ml volumetric flask and made up with distilled water. Then the absorbance of this solution was read at the λmax corresponding to the dye. From the standard calibration curve of the dye, the concentration of the dye in the discharge liquor was determined and dye uptake by the leather was quantified.

Conc.of dye in spent liquor (ppm)=
Conc.of dye by UV (ppm) ×Dilution factor

Amount of dye in spent liquor (mg)

=Concentration of dye in spent liquor(mg/ml)

×Volume of spent lliquor collected (ml)

Amount of dye absobed by the leather

=Amount of dye offered-Amount of dye in the spent liquor

$$\%\ Dye\ Absorbed = \frac{(Amount\ of\ Dye\ Absorbed)}{(Amount\ of\ Dye\ Offered)} \times 100$$

Physical Strength Characteristics of Crust Leathers

The four quadrant crust leathers were tested for physical strength properties. Sampling was done at respective symmetrical positions (near to the intersection) and conditioned as per standards ISO 2418:2005 and ISO2419:2005. Physical strength properties tensile strength and elongation at break, and double edge tear strength were measured as per standard procedure.[12]

Quantification of Color of the Leathers

The L, a, b values for the dyed crust samples (the four quadrants) were measured by color meter (Techkon Spectro Drive). And the color difference in terms of CIE value was calculated by making the control leather as reference.

Organoleptic Evaluations of Crust Leathers

The organoleptic properties such as softness, fullness, roundness, grain smoothness, grain tightness, and intensity of the shade of the color and overall appearance of the crust leathers treated with collagen hydrolysate samples (CHs) and control were evaluated by a group of experts. The values were rated from 1 to 10; higher value represents better functional property.

Results and Discussion

Trypsin Hydrolysis of Limed Trimmings

Limed trimming waste after deliming contains primarily collagen and these collagenous matrices (Type I) in their native form are stable against a wide range of proteases including trypsin. Hence, we have subjected the delimed collagenous matrix to denaturation by subjecting the matrix to temperature around 80°C for about 30 minutes. In thermally denatured form, a wide range of proteases can attack gelatinsed collagen. Zhang *et al* have carried out preparation of collagen hydrolysates using trypsin.[8] However they have subjected limed splits for reliming for 2 weeks and extracted gelatin at a temperature of 70 - 90°C, and then carried out trypsin hydrolysis to obtain collagen hydrolysate from gelatin. In our attempt to develop a simple and effective process for preparing collagen hydrolysate, we have strategized to use trypsin after subjecting the delimed collagenous matrix to thermal denaturation. The thermally denature delimed collagenous matrices were brought to temperature 37°C in order to have the matrix amenable for trypsin digestion. The amount of trypsin used for the enzymatic hydrolysis varied between 0.8 to 1.2% based on the delimed collagen matrix weight. In all the experimental trials the trypsinization was carried out for a period of 3 hours. The complete solubilization of delimed collagen matrix (denatured) was observed in all the cases. Trypsin generally breaks down the peptide bonds on the carboxyl side adjacent to the positive amino acid residues *viz*., lysine and arginine except when they are followed by proline. The collagen hydrolysates powder prepared from the enzymatically hydrolyzed delimed matrix was readily soluble unlike native collagen, which requires acidic or alkaline conditions for solubilization. Hence, the collagen hydrolysates prepared are suitable for retanning and the side chain functional groups of the fractions of collagen hydrolysate presents the possibility of extending good interaction with leather matrices during retanning processes. Earlier our group had prepared Type I collagen hydrolysate employing a similar methodology from bovine achilles tendons for biomedical

applications.[13] The collagen hydrolysates were observed to possess mild inherent anti-microbial property. It was of great interest to note the number of molecular fractions in the prepared collagen hydrolysate and their molecular weight ranges, whether these would support the purpose of retanning was further tested and reported here under.

Fast Protein Liquid Chromatography (FPLC)

Collagen hydrolysate prepared using 0.8% trypsin was characterized using FPLC analysis. The chromatogram of the collagen hydrolysates that had been separated using distilled water as mobile phase is presented in Figure 2. From the figure, we could observe about 6 fractions, of which 4 were major fraction and 2 were minor fractions. However any overlap of some of the similar molecular peptides within a fraction may not be ruled out. The integration of the six peaks with retention time at their respective peak areas of the chromatogram had been presented in Table I. The relative proportion of the first fraction was about 33%. The second and third fractions are also the major fractions accounting for about 30%. These fractions were eluted at 49, 55 and 60 minutes of retention time. Then the next higher proportion of the fractions was 14.88%. This fraction was obtained at 79 minutes of retention time.

Thus, based on the principle of size exclusion chromatography (SEC), about 80% of the collagen hydrolysates have relatively higher molecular weight with retention time of 49 to 66.67 minutes and 20% have relatively lower molecular weight with retention time of 71 to 126 minutes (Figure 2).

Collagen hydrolysates prepared by enzymatic hydrolysis with varied amount of trypsin *viz*., 0.8, 1.0 and 1.2%, their corresponding peak areas and the retention time is presented in Table II. The variation in the amount of enzyme did not result in any variation in the number of fractions of the collagen hydrolysates and the peak positons. However, slight variation in the distribution of high and low molecular weight fractions was observed between collagen hydrolysates prepared using 0.8, 1 and 1.2% of trypsin. With 0.8% trypsin, 80% of the fractions were observed to be of relatively high molecular weight and the proportion of the high molecular weight fractions slightly reduced by about 2% to 78% with increase in the trypsin amount from 0.8 to 1.0%. It will be of immense interest to note if these minor variations in the molecular fractions could lead to significant difference in the properties of the leathers retanned using these collagen hydrolysates.

Matrix Assisted Laser Desorption/Ionization (MALDI-TOF) of Collagen Hydrolysate

MALDI-TOF spectrum of the collagen hydrolysate sample prepared using 0.8% of trypsin is presented in Figure 3. MALDI-TOF spectrum clearly indicates the presence varied molecular weight fraction in the collagen hydrolysate sample. However, the

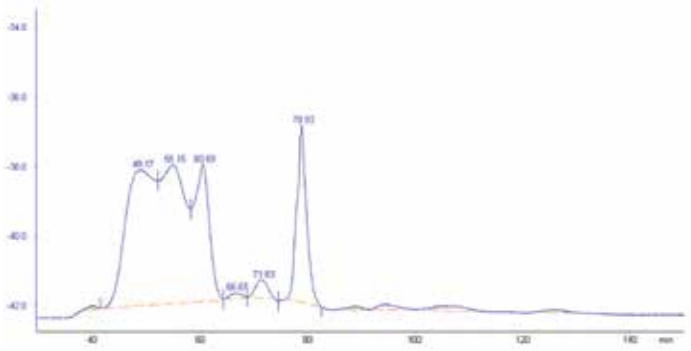

Figure 2. Chromatogram of collagen hdyrolysate, hydrolyzed using 0.8% of trypsin.

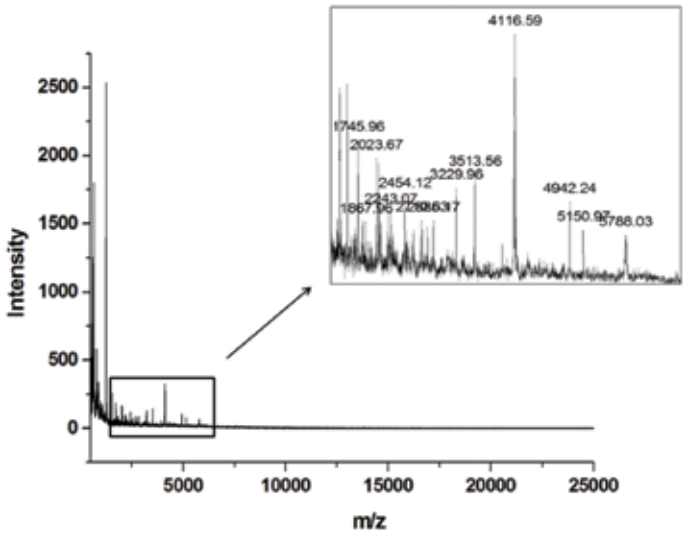

Figure 3. MALDI-TOF spectrum of collagen hdyrolysate, hydrolyzed using 0.8% of trypsin.

Table I
Relative proportions of collagen hydrolysate fractions obtained from limed trimmings enzymatically hydrolyzed using 0.8% trypsin.

Fraction No	Retention (min)	Area (mAU*min)	Relative proportions of each fraction (%)
1	49.17	25.67	33.49
2	55.15	22.08	28.8
3	60.69	13.75	17.94
4	66.67	1.23	1.62
5	71.63	2.5	3.27
6	78.93	11.41	14.88

molecular wight of the collagen hydrolysate fractions ranged predominantly between 1750 and 5800 daltons. These molecular weight fractions of collagen hydrolysates will not have much resistance for their diffusion into the leather matrix during post tanning processes and hence could fit in for retanning purposes. The molecular weight fractions obtained by Zhang et al was 10 – 50 kDa, whereas ours is less than 6 kDa, which would be ideally suitable for retanning applications.

Absorption of Collagen Hydrolysate

The different CHs for retanning, in view of their variations in molecular weight and size distribution are likely to bring about variation in the properties of the leathers. Hence, post tanning trials were conducted using CH-1, CH-2 and CH-3. In order to determine if there are variations in the uptake of CH-1, CH-2 and CH-3 used for retanning trials, collagen hydrolysate in the exhaust liquor had been determined based on the hydroxyproline content. The presence of hydroxyproline in the exhaust liquor is a direct contribution from collagen hydrolysate. The % collagen hydrolysate absorption by the leather is presented in Table III. It can be observed that CH-1, collagen hydrolysate that is digested (3 hours) with 0.8% trypsin, showed maximum absorption of about 86.5% compared to the other two CHs. It may be noted that collagen hydrolysates of CH-1 contained more composition of higher molecular weight fractions in comparison to CH-2 and CH-3. The presence of high proportion of high molecular weight collagen hydrolysate fractions have definitely augured well for the better uptake of CH-1 with wet blue leathers. Though uptake of collagen hydrolysate is marginally higher for CH-2 compared to CH-3, the difference between them is observed to be statistically insignificant. This data clearly signifies that the large molecular weight collagen hydrolysate fraction amongst the CHs is not restricted for diffusion at the conditions used for post tanning. Collagen hydrolysates carrying hydrophilic and hydrophobic amino acids and peptide backbone are likely to exhibit non-covalent interactions with the wet blue leathers. Collagen hydrolysates though higher or lower molecular weight fractions are likely to exhibit similar charge characteristics, because the distribution of amino acids between different fractions are likely to be similar. However, CH of large molecular weight fraction is most likely to have stronger interactions because of the presence of more functional groups and hence higher probability of interactions with the wet blue substrate. This could be a reason for better uptake of CH-1 in comparison to CH-2 and CH-3.

Percent Absorption of Dye

Leathers post tanned with collagen hydrolysate exhibited very good absorption of dye compared to control syntan. This was obviously perceived while processing in the drum; even before fixation process not much color was observed in the post tanning bath (Figure 4). The spent dye liquor also showed very high exhaustion of the dye compared to the leathers processed with control (Figure 5). The percent absorption of dye by the leather had been presented in Table IV, all CH experimental leather exhibited near zero discharge of dye in the wastewater. However, there had been no significant difference between the three CH experimental trials. Though collagen hydrolysates possess both positively and negatively charged functional groups, considering the pH 5.0 for post tanning, CH would exhibit weakly cationic charge and hence the leathers treated with collagen hydrolysates

TABLE II

Relative proportions of collagen hydrolysate fractions obtained from limed trimmings with varied amount of trypsin.

Retention time (min)	CH-1 (0.8% Trypsin)			CH-2 (1.0% Trypsin)			CH-3 (1.2% Trypsin)		
	Area (mAU*min)	% proportion	Proportion of High MW to Low MW	Area (mAU*min)	% proportion	Proportion of High MW to Low MW	Area (mAU*min)	% proportion	Proportion of High MW to Low MW
0 to 55.15	47.75	62.28		48.25	62.67		44.77	58.15	
55.15 to 60.69	13.76	17.94	80	12.32	16	78.67	13.99	18.17	76.31
60.69 to 71.63	3.75	4.89		4.11	5.33		3.01	3.91	
71.63 to 78.93	11.41	14.88	20	12.32	16	21.33	15.23	19.78	23.69
		100			100			100	

would have presented more functional groups for the interaction between leather matrix and dye. This could be a reason for better uptake of dye on to the leathers treated with collagen hydrolysates. The overall uptake of dye in the case of CH treated system was about 98%, which is 18% greater than the control retanning system.

Figure 4. Pictorial presentation of sample drums representing the uptake of CH treated and control leather samples during processing (i.e. before and after fixation).

Physical Strength Properties

The tensile strength, percent elongation and double edge tear strength had been presented in Table V. Experimental leathers had been observed to have better tensile strength, tear strength and elongation than the control. Though the control and samples are symmetrical there had been a difference in strength

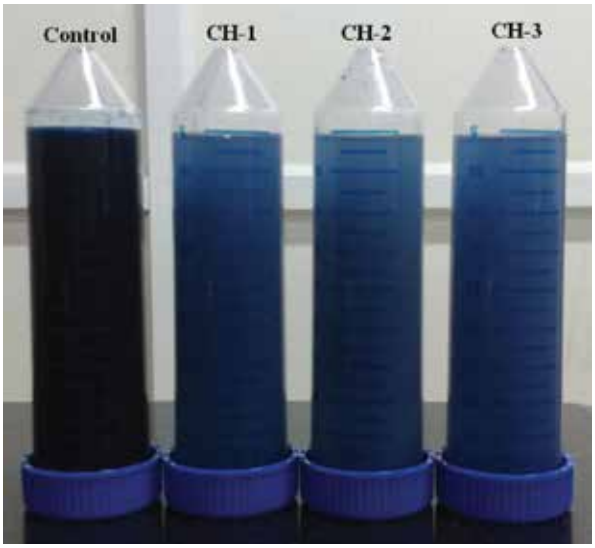

Figure 5. Pictorial presentation of spent liquors (after fixation) for CH and control.

Table III
Percent absorption of the collagen hydrolysate.

Retanning Agent	% CH absorbed by the leather
CH-1	86.58±0.65
CH-2	82.09±0.44
CH-3	81.21±0.28

Table IV
Percent dye uptake of the control and CH treated leathers.

Retanning	% Dye uptake
Commercial syntan	80.55±0.25
CH-1	98.41±0.20
CH-2	98.68±0.15
CH-3	98.32±0.10

properties due to collagen hydrolysate treatment. Leather samples from CH-1 showed relatively lower strength properties compared to CH-2 and CH-3. Although sampling had been taken closer to the intersection of four quadrants (Figure 1), samples CH-2 and CH-3 are closer to the butt region and hence locational influence for the strength may not be ruled out. However, the strength value of CH treated leathers was overwhelmingly higher compared to control leathers.

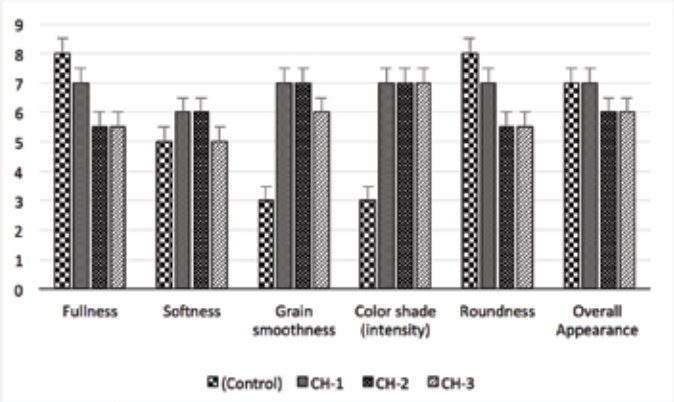

Figure 6. Organoleptic properties of leathers treated with CH and control.

Table V
Tensile and tear strength of control and CH treated leathers.

Retanning Treatment	Tensile Strength (N/mm^2)	Elongation (%)	Tear Load (N/mm)
Commercial syntan	19.37±1.15	63.13±1.32	49.21±1.08
CH-1	28.38±1.24	64.10±1.75	58.92±1.19
CH-2	33.29±1.11	68.07±1.43	64.98±1.26
CH-3	35.48±1.18	60.53±1.55	68.59±1.15

Table VI
L, a, b values of control and CH treated leathers.

Sample	L	a	b	ΔE
Control	39.96	-7.06	-20.1	-
CH-1	29.62	-4.14	-20.64	10.76
CH-2	26.94	-3.12	-19.03	13.65
CH-3	28.3	-3.43	-19.96	12.21

Quantification of Colors of the Leathers

The L, a, b values for the dyed crust samples (the four quadrants) and the color difference in terms of CIE value by making the control leather as a reference are presented in Table VI. From the table, **ΔE** value greater than five had been observed, clearly indicating significant difference in color shade between the control and the experimental leather samples. However, no significant difference in color observed amongst the experimental leathers.

Organoleptic Properties

The organoleptic properties of the experimental and corresponding control leathers are presented in Figure 6. The fullness and roundness properties of quadrant II (CH-1) are comparable with that of the control, whereas the grain tightness, grain smoothness, intensity of the shade/uniformity of the dyeing for quadrant II, III, IV, which has been treated with CH, are better than control leathers.

Collagen Hydrolysates: An Alternative Syntan

The important criteria for a retanning agent are to have appropriate molecular weight for diffusion in leather matrix and ideal functional groups for exhibiting interaction/binding with leather matrix. Collagen hydrolysates we prepared from limed trimming waste have the ideal distribution of molecular weight between 1750 and 5000 Da that would suit retanning application to achieve good penetration and distribution within the leather matrix. Furthermore, the multiple functional groups of the collagen hydrolysates make it amenable for interaction with the constituents of wet blue leather matrix, which clearly demonstrated through their very good exhaustion behavior. The leather matrix retanned with the collagen hydrolysate also exhibited higher dye uptake, which could be attributed to the increase in the functional sites available for binding of dyes. Hence, collagen hydrolysate can be classified as multifunctional syntan, which can provide bulk fullness and better color intensity due to higher dye uptake.

Conclusions

Collagen hydrolysates from limed trimming wastes were successfully prepared by a simple procedure using trypsin. The molecular weight fractions of the collagen hydrolysates prepared by the enzymatic hydrolysis were observed in the range of 1750 to 5800 daltons and a significant proportion were in the higher molecular weight range. This molecular weight range and the fraction proposition seem to fit in for retanning application.

Collagen hydrolysates significantly improve the exhaustion of dyes. About 98% of dye uptake can be achieved by treating the leathers with collagen hydrolysate. The data of tensile and tear strength indicate that collagen hydrolysate increases the strength

properties of the leathers. Furthermore, comparable fullness and better softness, grain smoothness and color shade of the grain are achieved by treating leathers with collagen hydrolysate. It can also concluded that collagen hydrolysate prepared using 0.8% trypsin resulted in better performance and properties to leathers.

Acknowledgements

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